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# UNDERSTANDING AGEING – THE ROLE OF MITOCHONDRIA IN DETERMINATION OF *CAENORHABDITIS ELEGANS* LIFE SPAN

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## ABSTRACT

Mitochondria are organelles found in eukaryotic cells. They are involved in many vital cellular functions. Consequently, mitochondrial dysfunction leads to a variety of human disorders. Many studies of the last 50 years showed that mitochondria are involved in the regulation of physiological ageing. However, the underlying mechanisms are still unknown. We aimed to analyze the mitochondrial role in ageing in *Caenorhabditis elegans* model system. Its short life cycle, powerful genetic tools and known fates of all 959 post-mitotic somatic cells make this nematode an excellent model system for ageing studies. Besides numerous advantages, the small body size of the worm brings along certain technical limitations. We developed a toolkit to analyze mitochondrial morphology, metabolic profile and electron transport chain (ETC) activities on a single-tissue level. In addition, we adapted a method for analysis of mtDNA copy number for use on individual animals.

Each mitochondrion has its own genome that is maintained by mitochondrial DNA polymerase gamma (POLG). By analyzing *polg-1* mutant worms that are deficient in the sole mitochondrial DNA polymerase, we showed that *C. elegans* mtDNA replication mainly takes place in the gonad, the only proliferative tissue in adult worms. Thus mtDNA depletion leads to marked dysfunction of this organ. Severe mtDNA depletion leads to embryonic arrest, whereas mild depletion does not affect development. We showed that mtDNA replication does not take place during embryogenesis; it starts during the L3 larval stage, correlating with germline proliferation. Taken together, mtDNA copies in the somatic tissues mainly stem from the oocyte and stay relatively unchanged during development and early adulthood. Remarkably, somatic tissues are not severely affected in *polg-1* deficient animals despite the marked overall mtDNA depletion in contrast to other model systems, namely flies and mice. Furthermore, we showed that mtDNA copy number exhibits substantial plasticity upon environmental stress.

Mitochondria are the major source of ATP, which they form by oxidative phosphorylation (OXPHOS). Defective OXPHOS often results in severe phenotypes or premature death in several animal models. However, studies in *C. elegans* showed that dysfunction in the mitochondrial respiratory chain is not necessarily lethal. It can rather result in lifespan prolongation in the so-called “Mit (mitochondrial) mutants”. We analyzed molecular mechanisms that underlie the longevity induced by

mitochondrial dysfunction. It has been shown that different mechanisms can affect the longevity of Mit mutants. We found that succinate dehydrogenase activity of electron transport chain (ETC) complex II (CII) influences the lifespan of Mit mutants independently of the insulin-like/IGF-1 pathway. We showed that mitochondrial unfolding protein response ($UPR^{mt}$) is up-regulated in both short- and long-lived Mit mutants. Furthermore, our results suggest that respiration rate is not necessarily linked to longevity. Analysis of several metabolic pathways in Mit mutants revealed that dysfunction of the mitochondrial respiratory chain leads to a common response characterized by up-regulation of the citric acid cycle, glycolysis, and some anaerobic pathways, accompanied by increase in neutral fat storage.

**Keywords: *C. elegans*, mitochondria, ageing, development, mtDNA copy number, metabolic changes, electron transport chain activities, succinate dehydrogenase.**

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## LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to by their Roman numerals.

**I.** ***Caenorhabditis elegans*** **as a model system for mtDNA replication defects**
Ivana Bratic, Jürgen Hench, Aleksandra Trifunovic
*Methods* 2010, 51:437-443

**II.** **Mitochondrial DNA level, but not active replicase, is essential for** ***Caenorhabditis elegans*** **development**
Ivana Bratic, Jürgen Hench, Johan Henriksson, Adam Antebi, Thomas R Bürglin and Aleksandra Trifunovic
*Nucleic Acid Research*, 2009, Vol.37, No.6:1817-1828

**III.** **A tissue-specific approach to the analysis of metabolic changes in** ***Caenorhabditis elegans***
Jürgen Hench, Ivana Bratic Hench, Claire Pujol, Sabine Ipsen, Susanne Brodesser, Arnaud Mourier, Markus Tolnay, Stephan Frank and Aleksandra Trifunovic
*Submitted Manuscript*
***J.H and I.B.H contributed equally to this work.***

**IV.** **Succinate dehydrogenase divergently regulates longevity in** ***Caenorhabditis elegans*** **mitochondrial mutants**
Ivana Bratic Hench, Claire Pujol, Marija Sumakovic, Jürgen Hench, Linda Baumann and Aleksandra Trifunovic
*Manuscript*

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ADP | Adenosine-di-phosphate |
| AMPK | AMP activated protein kinase |
| ATP | Adenosine-tri-phosphate |
| ATP8 | Mitochondrial DNA encoded subunit of complex V |
| bp | Base pair |
| CI | Complex I of the respiratory chain |
| CII | Complex II of the respiratory chain |
| CIII | Complex III of the respiratory chain |
| CIV | Complex IV of the respiratory chain |
| *clk-1* | Demethoxyubiquinone hydroxylase gene in *C. elegans* |
| Cox1 | Cytochrome oxidase subunit 1 |
| Cox2 | Cytochrome oxidase subunit 2 |
| cyt b | Cytochrome b of complex III gene |
| DAF-16 | Forkhead box O transcription factor in *C. elegans* |
| DAF-2 | Insulin/IGF-1 receptor in *C. elegans* |
| DNA | Deoxyribonucleic acid |
| DR | Dietary restriction |
| *eat-2* | A ligand-gated ion channel subunit gene in *C. elegans* |
| ETC | Electron transport chain |
| $FADH_2$ | Flavin adenine dinucleotide (reduced) |
| Fe-S | Iron sulfur cluster |
| FR | Fumarate reductase |
| *gas-1* | 49 kDa subunit of complex I gene in *C. elegans* |
| GFP | Green fluorescent protein |
| $H_2O_2$ | Hydrogen peroxide |
| HIF-1 | Hypoxia inducible transcription factor |
| IIs | Insulin signaling pathway |
| IMM | Inner mitochondrial membrane |
| IMS | Intermembrane space |
| ISP | Rieske iron sulfur protein of complex III |
| *isp-1* | Rieske iron sulfur protein of complex III gene in *C. elegans* |
| *mev-1* | Cytochrome b subunit of complex II (SDHC) gene in *C. elegans* |
| Mit | *C. elegans* mitochondrial mutants |
| mtDNA | Mitochondrial deoxyribonucleic acid |
| NADH | Nicotinamide adenine dinucleoitide (reduced) |
| *nuo-6* | NDUFB4/B15 subunit of complex I gene in *C. elegans* |
| $O_2$• | Superoxide radical |
| $O_H$ | Origin of replication of heavy strand |
| OH• | Hydroxyl radical |
| OMM | Outer mitochondrial membrane |
| OXPHOS | Oxidative phosphorylation |
| PHDs | Prolyl hydroxylases |
| POLG | DNA polymerase gamma |
| *polg-1* | DNA polymerase gamma gene in *C. elegans*, catalytic subunit |
| POLRMT | Mitochondrial RNA polymerase |
| RNA | Ribonucleic acid |
| RNAi | Ribonucleic acid interference |
| ROS | Reactive oxygen species |

| | |
|---|---|
| SDH | Succinate dehydrogenase |
| SOD1 | Copper zinc superoxidase dismutase |
| SOD2 | Manganese superoxidase dismutase |
| TCA | Tricarboxylic acid cycle, *syn.* citric acid cycle |
| $UPR^{mt}$ | Mitochondrial unfolded protein response |

# 1. Introduction

## 1.1. Mitochondrion - Power Plant of the Cell

A mitochondrion is a membrane-enclosed organelle found in most eukaryotic cells. Mitochondria are remarkably plastic and mobile organelles that constantly divide and fuse (Scott et al., 2003). The "endosymbiotic hypothesis" postulates that mitochondria originate from a single invasion of an archea-like host by a rickettsial group of α-proteobacteria ancestor that happened over 2 billion years ago (Doolittle, 1998; Martin & Müller, 1998). Each mitochondrion has two highly specialized membranes: the outer (OMM) and the inner mitochondrial membrane (IMM) that together form two compartments, the intermembrane space (IMS) and the mitochondrial matrix. The OMM is highly permeable for molecules smaller than 5kDa. Larger molecules are brought across the OMM by the translocase of the outer membrane (TOM complex) (Bohnert et al., 2007; Gebert et al., 2011). The IMM consists of a lipid bilayer containing cardiolipin making the IMM highly impermeable to ions and most molecules (Navarro & Boveris, 2007). Likewise, the IMM hosts transport proteins responsible for the transport of compounds needed for enzymes situated in mitochondrial matrix (translocase of the inner membrane – TIM or Oxa1) (Alberts et al., 2002). The IMS (space between the OMM and the IMM) is filled with small molecules (ions, sugars) that freely diffuse through the OMM. Thus the content of the IMS is highly similar to the cytosol (Herrmann & Neupert, 2000).

Mitochondria are often called "power-plants" since they convert various forms of energy to ATP through the process termed oxidative phosphorylation (OXPHOS). The respective enzymes are located in IMM infoldings called – based on their cross sectional appearance - *cristae* (*lat.pl.* crest, rooster's comb). Various metabolic pathways take place in the mitochondrial matrix: β-oxidation and tricarboxylic acid cycle (TCA, *syn.* citric acid cycle, Krebs cycle) (Alberts et al., 2002). Besides energy conversion, mitochondria are involved in a variety of different cellular processes: heme and iron-sulfur cluster biosynthesis, calcium homeostasis, cellular differentiation, cell death regulation and control of the cell cycle.

OXPHOS refers to the electron transport along the electron transport chain (ETC), situated in IMM, which is coupled to ADP phosphorylation, resulting in ATP production. The ETC proteins are grouped into four complexes (I-IV), which enable

electron flow from the electron donors (NADH and succinate) to oxygen, reducing it to water. The TCA produces NADH and $FADH_2$ that are the source of electrons for the ETC. Both NADH and $FADH_2$ are also synthesized in the cytoplasm through glycolysis and then imported into mitochondria by the malate-aspartate or the glycerol-phosphate shuttle system (Voet & Voet, 2004). The ETC receives electrons harvested from the TCA and fatty acid β-oxidation and transfers these electrons through a series of coupled reduction-oxidation reactions until finally molecular oxygen is reduced to water. The ETC complexes I, III and IV function as proton pumps that build up a proton gradient across the IMM, a so called chemiosmotic potential or proton motive force, which enables ADP phosphorylation via the ATP synthase (complex V - $F_oF_1$ ATPase) (Reid et al., 1966). The rate of mitochondrial respiration depends on the phosphorylation potential reflected by the ratio between ATP and ADP/phosphate ([ATP]/[ADP][Pi]) that is regulated by the adenine nucleotide translocase (ANT). During increased cellular energy demands, when more ADP is available and the phosphorylation potential is decreased, the respiration rate is increased, resulting in increased ATP synthesis. When oxygen is limited, glycolytic products are metabolized in the cytosol in a less efficient anaerobic manner independent of mitochondria.

Due to the pivotal role of mitochondria in maintaining cellular homeostasis, it is rather expected that mitochondrial dysfunction is a cause of a wide range of human diseases. While many mitochondrial defects result in early, often intrauterine or postpartal death due to their effect on metabolism, there are other forms that cause tissue degeneration later in life. The latter group of diseases includes neural and muscular degeneration. Besides these mitochondrial diseases, many other types of more common disorders such as certain forms of diabetes mellitus, neoplasms, neurodegenerative disorders (e.g. Parkinson, Huntington, Alzheimer), hypoxic damage or fatty liver disease involve mitochondrial malfunction.

## 1.2. Mitochondrial Genome and its Structure

Mitochondria contain their own independent genome (mtDNA). In humans, each cell possesses 1000-10.000 mtDNA copies. In yeast mitochondria, mtDNA molecules are organized in nucleoids that consist of one to two mitochondrial genomes associated with different polypeptides. In vertebrate, alike in yeast, mtDNA

molecules are organized in nucleoids containing mtDNA and different proteins (Wang & Bogenhagen, 2006). In variety of human cells, nucleoids are approximately 70nm in size and contain in average 1.4 mtDNA molecules (Kukat et al., 2011). However, other estimates of the amount of mtDNA molecules per nucleoid and its size also exist (Garrido et al., 2003; Wang & Bogenhagen, 2006). Mitochondrial nucleoids are also genetic units that seem not to exchange mtDNAs between each other (Gilkerson & Schon, 2008).

The strands of the DNA duplex are distinguished as "heavy" (H) and "light" (L) strand on the basis of their G+T base composition and different densities in denaturing cesium chlorides gradients. The mitochondrial genome is characterized by dense gene packing, relaxed codon usage and variant genetic code (Alberts et al., 2002). Dense gene packing is explained by the lack of introns and by presence of a short non-coding region called D-loop. In mammalian mtDNA the origin of heavy strand replication ($O_H$) and promoters for both H- and L- strand (HSP and LSP) transcription are located in the D-loop. The codon usage differs from the "universal code" leading to difference in 4 out of 64 codons (Alberts et al., 2002).

The mtDNA genome shows notable variation in shape between species. While most have circular mtDNA, some linear variants exist as well (e.g. in *Cnidaria*, *Saccharomyces sp.* and plants) (Nosek et al., 1998). The size also varies. While mtDNA in different vertebrate species ranges from 16 to 18 kb, mtDNA genome of *Caenorhabditis elegans* is approximately 13.8 kb in size (Anderson et al., 1981; Lemire, 2005) (Figure 1). mtDNA gene content and gene order similarly vary between species, most likely due to gene transfer from mitochondrion to the nucleus (Gray et al., 2001). Thus, human mtDNA encodes for 13 proteins, 2 rRNAs and 22 tRNA genes, whereas *C. elegans* mtDNA genome lacks ATP8 subunit of the complex V (CV) (Gray, 1999; Lemire, 2005) (Figure 1).

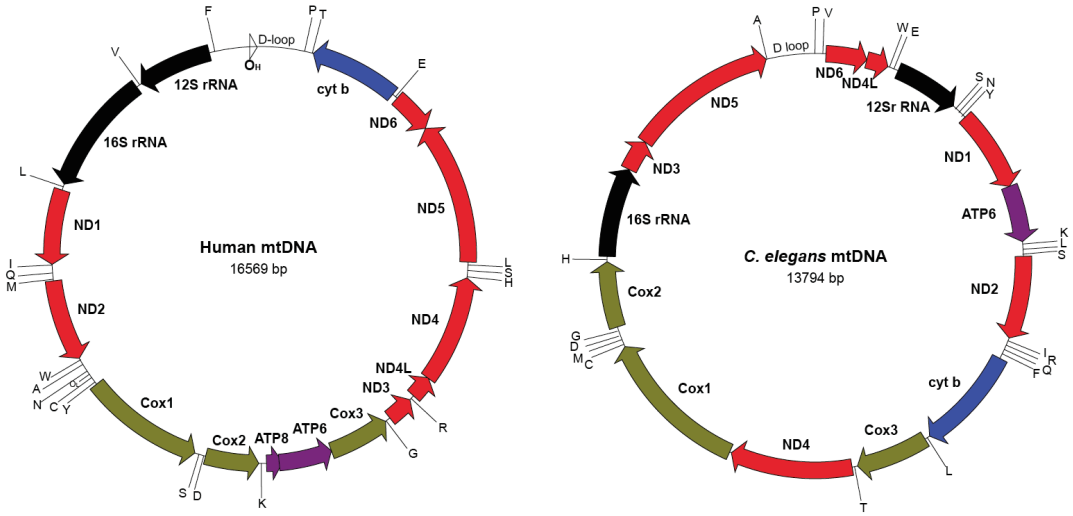


**Figure 1. Gene organization in human and *C. elegans* mtDNA.** The human mtDNA encodes for 13 protein coding genes (ND1-ND6, ND4L, cyt b, Cox1, Cox2, Cox3, ATP6 and ATP8), 2 rRNAs (16s rRNA and 12S rRNA) and 22 tRNAs. *C. elegans* mtDNA differs by the lack of the ATP8. The tRNA genes are labeled by the single letter code. Images adapted from (Garesse, 2001; Lemire, 2005).

Mammalian mtDNA encodes for seven subunits of complex I (ND1-ND6, ND4L), one subunit of complex III (cytochrome b/cyt b), three subunits of complex IV (Cox1, Cox2 and Cox3) and two subunits of complex V (ATP6, ATP8). 13 genes encode ETC subunits, 14 tRNAs and both rRNAs are transcribed from the H-strand, while one mRNA (for ND6) and 8 tRNAs from L-strand (Anderson et al., 1981). Of all ETC, CII is exclusively encoded by nuclear DNA (Figure 1).

## 1.3. Replication of the Mitochondrial Genome

The mode of mtDNA replication is subject of intense debate. The first model – the *strand displacement model* – was postulated in 1972 and proposes that replication starts from $O_H$ situated in the D-loop, downstream of LSP. Transcription from the LSP promoter generates the RNA primer needed for mitochondrial DNA polymerase gamma (POLG) to initiate the replication (Brown et al., 2008). Leading-strand (H-strand) replication proceeds unidirectional with displacement of the parental H strand until approximately 60% of mtDNA is copied, giving rise to a daughter H-strand. The origin of replication of the L-strand ($O_L$) is located in a short 30 bp spacer region

flanked by five tRNA (WANCY) genes (Figure 1). When the replication fork passes $O_L$, the parental H-strand becomes as a single strand with the potential to form a stable stem-loop structure. This loop enables replication of the L-strand to start and proceed in the opposite direction. Replication of the L-strand requires synthesis of the RNA primer by the mitochondrial RNA polymerase (POLRMT) (Fusté et al., 2010; Wong & Clayton, 1985a, 1985b, 1986). Replication of mtDNA according to *strand displacement model* is asynchronous and continuous on both strands, resulting in two daughter molecules. The separation of daughter molecules occurs before the light strand synthesis is finished resulting in one fully replicated daughter molecule (α daughter) and one partially replicated which will latter form fully replicated daughter molecule (β daughter) (Robberson & Clayton, 1972; Robberson et al., 1972).

The second model for mtDNA replication – the *strand coupled replication* - was only recently proposed (Holt et al., 2000; Pohjoismäki et al., 2010; Yang et al., 2002). This model is mainly derived from data based on nuclease treatment of mtDNA and 2D-gel electrophoresis. It was proposed that this mode of replication is more common in animal tissues and cells recovering from a depletion of mtDNA (Holt et al., 2000). According to this model, mammalian mtDNA replicates via coupled synchronous synthesis of leading (H) and lagging (L) strand that is initiated from a zone near $O_H$ followed by progression of two replication forks around the mtDNA circle. As opposed to the *strand displacement model*, this model suggests that L-strand replication initiates at multiples sites via synthesis of short Okazaki fragments (RITOLS intermediates) (Yasukawa et al., 2006).

Present data speak in favor of both models. Thus, there is need for better understanding of the mtDNA replication mechanism to test whether both modes occur in parallel or if the proposed models are the result of experimental artifacts.

### 1.4. mtDNA Replication Machinery

mtDNA synthesis randomly occurs throughout the cell cycle and is independent of nuclear DNA replication (Bogenhagen & Clayton, 1977). Not all mtDNA molecules are necessarily replicated (Clayton, 1982). Nuclear genes encode the mtDNA replication machinery and resulting proteins are imported into mitochondria. In contrast to nuclear DNA, mitochondrial DNA transcription and replication are coupled events. In vertebrates, POLG is a sole DNA polymerase devoted to mtDNA

synthesis. Further components such as single stranded DNA binding proteins (mtSSB), helicase (TWINKLE), mitochondrial transcription factor A (TFAM), RNA polymerase (POLRMT), RNA processing enzymes (RNaseH1) and topoisomerase (mtTOP1) are also required for mtDNA replication (Falkenberg et al., 2007).

TFAM actively participates in bending and packaging of mtDNA into nucleoids, analogous to histones in nuclear DNA. Thus, it has a vital role in maintaining mtDNA integrity (Gilkerson & Schon, 2008). During mtDNA replication, TWINKLE unwinds a 20 bp DNA duplex with 5'-3' polarity in ATP-dependent manner. The resulting ssDNA wraps around mtSSB by which the rate of TWINKLE-dependent mtDNA unwinding is further stimulated (Falkenberg et al., 2007). mtSSB proteins function in helix destabilization and enhancing the activity and processivity of POLG (Farr et al., 1999). POLRMT has a role in primer synthesis required for the initiation of lagging-strand DNA synthesis. RNaseH1 removes the short RNA primers used for the initiation of H- and L-strand DNA synthesis. Lastly, mTOP1 relaxes negative supercoils and it removes positive supercoils at the replication fork that result from DNA helicase action (Falkenberg et al., 2007).

*mtDNA POLG:* POLG belongs to the family A group of DNA polymerases prototypically represented by *Escherichia coli* DNA polymerase I. It was first identified as an RNA dependent DNA polymerase in human HeLa cells (Fridlender et al., 1972). In mammals POLG is an asymmetric heterotrimer of one catalytic subunit (POLG-A) and two accessory subunits (POLG-B). The POLG-A catalytic subunit has a molecular mass of 140 kDa and exhibits 5'-3' polymerase, 3'-5' exonuclease and 5'-deoxyribose phosphate lyase activity (Falkenberg et al., 2007). Polymerase domain contains three conserved motifs termed A, B and C. The crystal structure of human POLG revealed presence of a spacer domain, between the polymerase and exonuclease domain. This spacer domain is responsible for the processivity (the number of nucleotides added by a DNA polymerase per association/dissociation with the template) of DNA synthesis (Falkenberg & Larsson, 2009; Lee et al., 2009).

Human POLG-B accessory subunit has a molecular mass of 55 kDa and shares structural similarity with IIa class of aminoacyl tRNA synthetases. The crystal structure of human mtDNA POLG showed that only one of the accessory subunits forms extensive contacts with the POLG-A monomer. POLG-B is not required for DNA polymerase activity, but it is critical for mtDNA maintenance. The accessory subunit also increases the processivity and activity of the catalytic subunit by

affecting the conformation of POLG-A. POLG-B additionally accelerates the polymerization rate during DNA synthesis, by increasing the number of nucleotides incorporated per binding event, and simultaneously decreases the exonuclease activity of the catalytic subunit (Lee et al., 2009). POLG-B subunit has been identified in human, mouse, frog and fruit fly, however it has not been identified in the genomes of *Saccharomyces cerevisiae* and *C. elegans*. This might be due to the horizontal gene transfer from a bacterium to the lineage of higher animals (Lucas et al., 2004). Most likely, the ancestor of human POLG-A first acquired a spacer domain in the catalytic subunit, which then allowed a POLG-B like protein to interact. Interacting domains might subsequently co-evolve to increase the processivity of DNA synthesis (Lee et al., 2009).

Fidelity of DNA replication is achieved through selection of correct nucleotide during polymerization process, error correction via 3'-5' exonucleolytic editing (which removes mismatches at the 3' end of the growing DNA chain) by POLG and by mismatch repair. The exonuclease domain is characterized by three highly conserved motifs - Exo I, Exo II and Exo III - located in the N-terminal part of the protein. While mismatch repair mainly contributes to removal of transition mutations, exonucleolytic-proofreading activity of POLG mainly removes A to T transversions (Vanderstraeten et al., 1998). The decision to excise is made at the polymerase site. Upon mismatch, polymerase function stalls and reaction kinetic alters, favoring exonuclease removal instead of forward polymerization (Johnson & Johnson, 2001).

*POLG and its Role in Human Diseases:* To date approximately 147 disease associated mutations in POLG have been described, many of which map to catalytic and linker domain of the POLG-A subunit. Only few mutations have been found in POLG-B (Copeland, 2008). Most disease phenotypes linked with POLG mutations are associated with mtDNA depletion and/or accumulation of mtDNA mutations and deletions. Majority of the mutations were identified as compound heterozygotes where each POLG allele has one or more different mutations (Copeland, 2008). Mutations in POLG lead to a wide spectrum of neurological diseases such as Alpers syndrome, autosomal dominant or recessive external ophthalmoplegia (PEO) and ataxia-neuropathy syndromes (Stumpf & Copeland, 2011). Alpers syndrome is usually fatal during first years of life. Patients with Alpers syndrome suffer from psychomotor retardation, seizures and liver failure due to severe mtDNA depletion (Nguyen et al., 2005). PEO manifests in adulthood and is characterized by muscle

weakness, exercise intolerance, sensory ataxic neuropathy and respiratory failure due to muscle fatigue (Stumpf & Copeland, 2011). Ataxia neuropathy, also termed mitochondrial-associated ataxia syndrome (MIRAS), onsets in the 2nd to 4th decade, and is characterized by peripheral neuropathy, dysarthria, mild cognitive impairment, involuntary movements, psychiatric symptoms, myoclonus and epileptic seizures (Copeland, 2008). In addition, dominant POLG mutations are implicated in male infertility and some multisystem disorders such as Parkinsonism and premature menopause, which are not typical of mitochondrial disease (Luoma et al., 2004; Rovio et al., 2001).

## 1.5. Mitochondrial Inheritance

The mtDNA replication is not linked to the cell cycle, and some mtDNA molecules are replicated multiple times while others are not replicated at all (Clayton, 1982). This makes it possible for a single mutation to clonally expand or to be lost at cell division, thus leading to profoundly different levels of mutated mtDNA in individual cells. Considering that mitochondria are polyploidy, the status of homoplasmy or heteroplasmy is potentially reached. The status of homoplasmy refers to a cell that solely contains identical mtDNA copies. Once mtDNA copies are not identical anymore, cell's status is termed 'heteroplasmy'. When the heteroplasmic mtDNA, carrying pathogenic mutations, is present in a cell during embryogenesis, this can lead to variety of pathological phenotypes in mammals, and it can affect almost all tissues (Larsson, 2010). In mammals, mtDNA is strictly maternally inherited and the heteroplasmy levels in the offspring often differs from the one found in the mother. The fraction of mutated mtDNA molecules has to exceed a certain threshold in order to cause respiratory chain dysfunction (Figure 2). The threshold depends on mutation (ranging from 90% for some tRNA mutations to 60% for mtDNA deletions) and tissue type (Larsson & Clayton, 1995; Trifunovic, 2006). High heteroplasmy of a pathogenic mtDNA mutation often leads to a mosaic pattern of respiratory-chain deficiency. The amount of defective cells in a tissue or organ determines whether mitochondrial disease becomes apparent or not (Stewart et al., 2008).

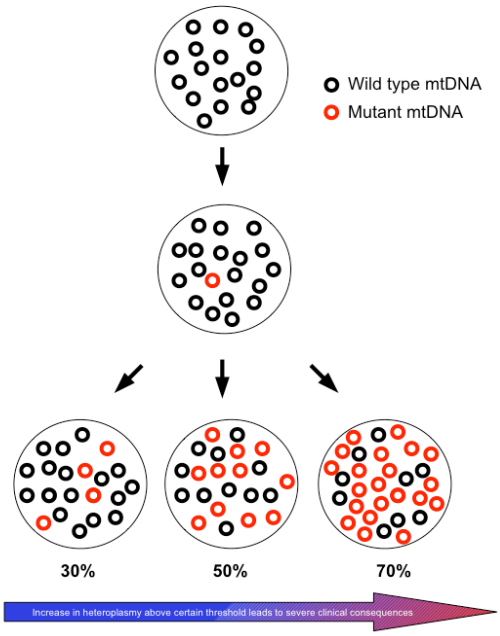


**Figure 2. Clonal expansion of mutated mtDNA molecules.** Mitotic segregation of mtDNA can lead to loss of a somatic mtDNA mutation in some cells and clonal expansion in others. The fraction of mutated mtDNA molecules has to reach a certain minimal threshold level to cause disease (Larsson, 2010).

However, the most deleterious mtDNA mutations get eliminated from the female germ line via a strong purifying selection in combination with a genetic "bottleneck" for mtDNA transmission (Stewart et al., 2008). The mitochondrial bottleneck hypothesis aimed to explain the change in heteroplasmy levels observed within one generation. Pedigree analyses of heteroplasmic cows showed that mtDNA genotypes shift rapidly among offspring and return to homoplasmy in some progeny within two or three generations. This suggests that the mtDNA bottleneck that occurs during early embryogenesis could account for a rapid mtDNA segregation (Ashley et al., 1989; Laipis et al., 1988). However, the molecular mechanism underlying the purifying selection still remains unknown. There are three hypotheses postulated to explain mtDNA genetic bottleneck. First proposes that a variation in heteroplasmy levels results from unequal partitioning (segregation) of mutant and wild type mtDNA genomes during cell division. The drift in heteroplasmy levels occurs as a consequence of the reduction of mtDNA copy number prior to expansion of primordial germ cell population (Cree et al., 2008). Second suggests that unequal segregation of homoplasmic nucleoids in PGCs is the driving force of the mtDNA genetic bottleneck (Cao et al., 2007, 2009). Lastly, genetic bottleneck is explained by

the preferential replication of a subpopulation of the available mtDNA genomes during oocyte maturation in post-natal life (Wai et al., 2008).

To date, a conclusive answer to the question of how bottleneck for mtDNA segregation occurs and where is temporally and spatially located within the mammalian reproductive cycle still awaits further experimentation.

## 1.6. Respiratory Chain

The electron transport chain consists of the four respiratory enzyme complexes (designated I-IV), located in the IMM. Electrons are extracted from NADH at complex I (CI), from $FADH_2$ at CII and then transported by ubiquinone or coenzyme $Q_{10}$ (CoQ) to the complex III (CIII). From CIII, electrons are donated to cytochrome c, which in turn passes electrons to complex IV (CIV). At the CIV, electrons will reduce molecular oxygen to form water (Figure 3). As electrons are passed along the ETC the released energy builds up a proton gradient across the membrane, which drives ATP synthesis via CV or ATP synthase. Electron transport chain complexes (I-IV), together with CV represent the OXPHOS system. The oxygen consumption and OXPHOS are tightly coupled in healthy mitochondria. The coupling between respiration and ATP synthesis in mitochondria was postulated in 1961 and is also referred to as the chemiosmotic theory (Mitchell, 1961). OXPHOS capacity varies among different tissues. This depends on differences in the amount, stoichiometry and maximal activity of its components. Mammalian heart and skeletal muscle are characterized with the highest OXPHOS capacity and low resistance to OXPHOS malfunction, while liver and kidney have a lower OXPHOS capacity and lower sensitivity to OXPHOS defects (Koopman et al., 2010). To date, two models explain the organization of ETC complexes in the IMM. The *random diffusion model* or *fluid model* proposes that ETC complexes diffuse freely within the IMM and electrons flow between them, via ubiquinone and cytochrome c mobile carriers. Transfer would occur by random collision of the involved components (Hackenbrock et al., 1986). The *solid model* proposes that ETC complexes are organized in so-called supercomplexes within IMM, which perform a fast and efficient electron transfer. In many organisms, CI, III and IV were found to form such supercomplexes. Cardiolipin, a diphosphathidiylglycerol lipid in the IMM, seems to be necessary for supercomplexes formation (Acín-Pérez et al., 2008; Boekema & Braun, 2007;

Dudkina et al., 2010).

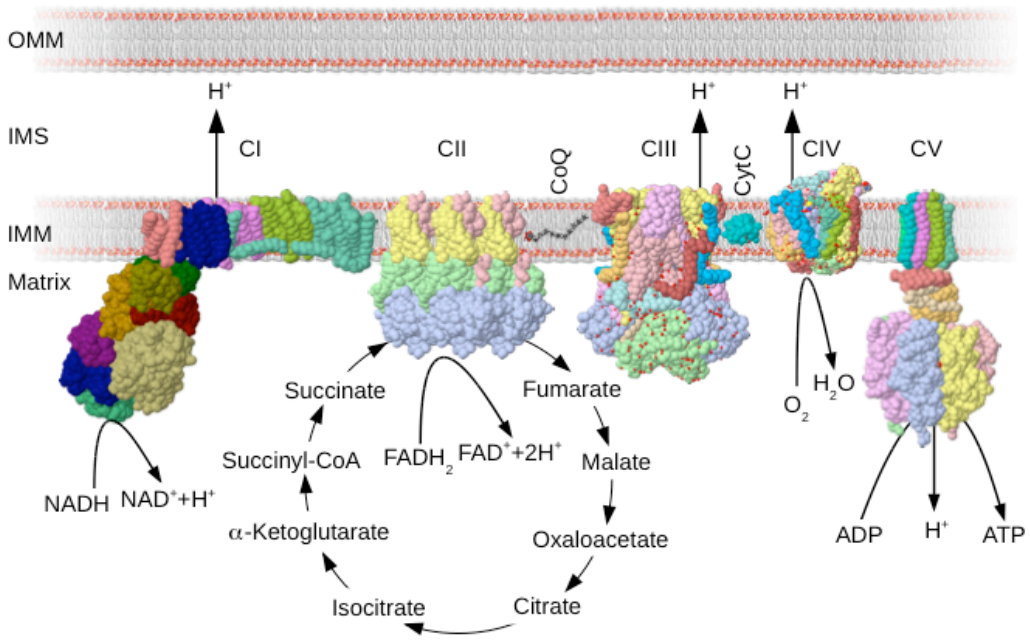


**Figure 3. Respiratory chain.** Complex I-V situated in the IMM. All complexes except CII translocate protons. CII, in addition to electron transfer, have a role in the TCA cycle. Crystal structures of ETC complexes and cytochrome c are retrieved from Protein Data Bank database (entries: 3M9S, 2WDV, 1NTM, 3ASN, 2XND, 3CYT).

*Complex I* (NADH ubiquinone oxidoreductase, EC 1.6.5.3) is the largest ETC complex and measures over 900 kDa. CI couples oxidation of NADH to the reduction of ubiquinone. Electrons from reduced NADH are abstracted at the matrix face of CI. During this process, electrons can escape from CI and react with ambient oxygen, producing superoxide. The mammalian CI contains 45 different subunits of which seven are mtDNA encoded (Carroll et al., 2006; Lemmagray et al., 2008). Electron microscopy analysis proposes that CI has an L-shaped conformation with a peripheral arm located in the matrix and an IMM-embedded arm (Koopman et al., 2010). CI can be subdivided into three modules: N, responsible for NADH oxidation; Q, involved in CoQ reduction and P, involved in proton pumping (Koopman et al., 2010). CI activity can also be influenced by various drugs that mainly act at the terminal electron donor step, inhibiting CoQ reduction (Koopman et al., 2010). Inhibition of CI by rotenone (also used as insecticide), that specifically block ubiquinone binding site, can induce Parkinsonism, including characteristic pathomorphological changes, suggesting a role

of CI in this nerodegenerative disorder (Betarbet et al., 2000).

*Complex II* (succinate:ubiquinone reductase or succinate dehydrogenase, EC 1.3.5.1) is the only ETC complex that is entirely encoded by nuclear DNA. Beside its role in OXPHOS it also participates in the TCA cycle (Figure 3). CII is the only ETC complex that does not translocate protons across IMM, but only feeds electrons to the electron transport chain. In mammals, it is a trimer of heterotetramers where each tetramer consists of two catalytic hydrophilic subunits, a flavoprotein (SDHA) and iron sulfur protein (SDHB) and two hydrophobic membrane anchoring subunits, SDHC and SDHD, that link the catalytic subunits to the matrix side of the IMM. The SDHA subunit has FAD and substrate binding sites. SDHB contains three FeS clusters ([2Fe-2S], [4Fe-4S] and [3Fe-4S]) needed for electron transfer to ubiquinone. Ubiquinone, electron carrier, is located in a cleft composed of SDHC and SDHD subunits close to the [3Fe-4S] cluster of SDHB surrounded with highly conserved amino-acids (Yankovskaya et al., 2003). CII couples the oxidation of succinate to fumarate in the mitochondrial matrix with the reduction of ubiquinone in the IMM using the cofactor flavine adenine nucleotide (FAD). Oxidation of succinate to fumarate results in transfer of electron to the FAD cofactor, then electron is transferred to iron-sulfur clusters situated in SDHB subunit, which pass the electron through the protein towards ubiquinone molecule bound to SDHC and SDHD subunits (Saraste, 1999). Malonate and oxaloacetate are succinate analogues that inhibit CII via binding to the dicarboxylate binding site (Dervartanian & Veeger, 1964; Dröse et al., 2011). Interestingly, the majority of the CII seems not to be part of ETC supercomplexes, probably due to its role in the TCA cycle. Still, a significant portion of complex II associated with CI, III and IV was detected (Acín-Pérez et al., 2008; Dudkina et al., 2010).

*Complex III* (ubiquinol-cytochrome c reductase, EC 1.10.2.2) delivers electrons from reduced ubiquinone (ubiquinol) to cytochrome c. This redox reaction is coupled to proton translocation across the IMM from the mitochondrial matrix to the IMS by the Q cycle (Saraste, 1999). CIII is a dimer of 248 kDa and each monomer consists of eleven subunits, among whose only three carry redox centers for electron transfers: cytochrome b with two heme groups that have different redox potentials, designated $b_H$ and $b_L$ (high and low), a membrane anchored iron sulfur protein (ISP) that carries a Rieske-type center ($F_2S_2$) and a membrane anchored cytochrome c1. In mammals, cytochrome b is the only mtDNA encoded subunit of CIII (Iwata et al., 1998; Xia et

al., 1997; Zhang et al., 1998). Electron flow from heme $b_H$ to ubiquinone can be specifically blocked with antymicin A (originating from *Streptomyces sp.,* also used as a fish poison), while myxothiazol (from the bacterium *Myxococcus fulvus*) stops the electron flow from ubiquinol to the iron sulfur center and indirectly also to heme $b_L$ (Xia et al., 1997).

*Complex IV* (cytochrome c oxidase, 204kDa, EC 1.9.3.1) is a homodimer. Its monomers consist of 13 subunits of which the three largest are mitochondrial encoded (Cox1, Cox2, Cox3). These three subunits form the core of the enzyme surrounded by 10 nuclear encoded subunits. Cox1 and Cox2 contain two heme A groups (a and $a_3$) and two copper centers ($Cu_A$ and $Cu_B$) responsible for the electron transport. Cox3 may function as a proton pump through the IMM. CIV couples reduction of $O_2$ to $2H_2O$ with proton pumping across the IMM. Soluble cytochrome c donates electrons to $Cu_A$ metal center of Cox2. Electrons are then subsequently transferred to the cytochrome a in Cox1 and then to the bimetallic cytochrome $a_3/Cu_B$ active site in Cox1. Two hydrophilic channels, D and K, connect the active site to the aqueous phase of mitochondrial matrix. The reduction of oxygen at the active site of Cox1 is coupled with translocation of four protons from the matrix side, two for the reduction of oxygen into water and two across IMM to the IMS (Saraste, 1999; Tsukihara & Yoshikawa, 1998; Tsukihara et al., 1996).

*Complex V* ($F_1F_0$ ATP synthase, 500kDa, EC 3.6.3.14) uses the energy of the proton gradient, generated across the membrane by CI-CIV, to drive synthesis of ATP from ADP and inorganic phosphate. The bovine CV has 16 subunits of which two are encoded by mitochondria (ATP6, ATP8). It consists of a hydrophobic, membrane-embedded domain $F_0$ and a hydrophilic, globular domain $F_1$ located in the mitochondrial matrix (Elston et al., 1998). $F_1$ contains the binding sites for ADP and Pi and acts as the catalytic ATP synthase domain. Energy released by proton flux through the $F_0$ is relayed to the catalytic sites of the $F_1$ domain. For each ATP synthesized three protons are used. CV can also hydrolyze ATP to pump protons against an electrochemical gradient (Abrahams et al., 1994). Each F1 unit contains, among others, three catalytic β subunits. Each catalytic site passes constantly through three states: open - unbound, loose - bound ADP and Pi and tight -bound ATP. Energy is required for substrate binding and for release of ATP, but not for ATP synthesis (Saraste, 1999). Oligomycin (a makrolid from *Streptomyces sp.*) and dicyclohexylcarbodi-imide inhibit CV activity by interaction with $F_0$ (Lutter et al.,

1993). Oligomerization of dimeric ATP synthases is essential for cristae formation (Couoh-Cardel et al., 2010; Dudkina et al., 2005).

## 1.7. Generation of Reactive Oxygen Species (ROS)

ROS are chemically reactive molecules containing oxygen (O2•, $H_2O_2$, OH•) that are byproducts of regular metabolism. However, ROS are not only a waste of OXPHOS, but are also signaling molecules involved in cellular stress response. During respiration, electron leakage to oxygen could lead to superoxide production (O2•). O2• causes damage to proteins, lipids and DNA. The cellular antioxidative protection system eliminates ROS produced during respiration. O2• is converted to hydrogen peroxide ($H_2O_2$) via superoxide dismutases (SOD). There are several types of SOD in mammalian cells: Cu/Zn SOD in the cytosol and IMS (SOD1), Cu/Zn SOD in the extracellular matrix (EC-SOD, SOD3) and Mn/SOD (SOD2) in the mitochondrial matrix (Nozik-Grayck et al., 2005). O2• reside in the mitochondria, while $H_2O_2$ can freely diffuse through the membrane out of mitochondria (Han et al., 2001). $H_2O_2$ is transformed to water and oxygen by glutathione peroxidase present in the cytoplasm and mitochondria or via catalase in peroxisomes. If $H_2O_2$ is not removed, it can react with reduced transition metals (e.g. iron) via Fenton's reaction and form highly reactive hydroxyl radicals (OH•) that cause lipid peroxidation (Kowald, 2001). The sites with the maximum rates of O2• production within ETC are CI and CIII. CI produces O2• into mitochondrial matrix, whereas CIII to mitochondrial matrix and IMS (Brand, 2010). Other ROS producing sites exist in the cell, but their role in ROS production under physiological conditions still remains to be determined.

Besides antioxidant enzymes, non-enzymatic molecules are part of the cellular antioxidative defense system. The two main antioxidants in the hydrophilic compartment are gluthatione (GSH) and ascorbate, while vitamin E and carotenoids act in lipophilic environments. Vitamin E acts on lipid peroxyl groups, while carotenoids (e.g. coenzyme Q) quench singlet oxygen (Sanz et al., 2006).

## 1.8. What is ageing?

*Ageing is a deleterious, progressive phenomenon in an organism and all individuals that do not succumb due to accidental death will be affected in species that age* (Strehler, 1982).

This is just one of the numerous definitions of the ageing process. Ageing is still one of the least understood processes in animal biology. Characteristic signs of ageing in humans are a gradual decline in height and weight due to loss of muscle and bone mass, a lower metabolic rate, a decline in memory, sexual activity, olfaction, vision and exercise performance. Interestingly, some animal species have exceptionally long life as they can live for centuries (e.g. certain sponges and corals live more than 200 years) (Bergquist et al., 2000). For a long time it was thought that ageing was a passive process of deterioration that occurred in haphazard way. Numerous, more recent studies suggest that ageing is actively regulated by signaling pathways and transcription factors (Kenyon, 2010b). The introduction of the nematode *C. elegans* as a suitable model for analysis of lifespan modification allowed for pioneering work that led to exciting findings in ageing research (Johnson, 1990). At present, a large number of genes and molecular pathways have been connected to ageing control in *C. elegans*, *D. melanogaster* and mammalian model systems. The involved genes regulate different cellular functions that gave rise to the idea that ageing is programmable and highly complex. Certainly, the genetic background is not the only determinant of lifespan, as seen in monozygotic human twins that usually differ in lifespan (Kirkwood, 2005a; Sadler et al., 2011). The nematode *Caenorhabditis briggsae* was a pioneer model for ultra-structural studies of age-associated cellular changes (Epstein & Gershon, 1972; Epstein et al., 1972). Progressive, age-dependent accumulation of electron dense granules that contain acid phosphatase activity in intestinal cells, and eventually lead to decay of this tissue, was the major change observed in *C. briggsae*. This change was more prominent than the originally expected neuromuscular degeneration (Epstein et al., 1972). Later, similar changes were observed in *C. elegans* (Herndon et al., 2002).

There are many attempts to explain ageing as a cause of either failure of genetic replication (telomere loss theory), trade-off between reproduction and repair mechanisms (disposable soma theory), mitochondrial dysfunction and increased oxidative stress (mitochondrial theory) or accumulation of damaged proteins (waste

accumulation theory) (Kirkwood, 2005b; Soltow et al., 2010). Likely ageing involves not only one of these processes, but rather their combination results in accumulation of wide variety of molecular and cellular damage.

### *1.8.1. Free Radical / Mitochondrial Theory of Ageing*

The free radical theory of ageing postulates that the accumulation of oxygen free radicals produced during normal metabolism in the cell causes oxidative damage to cellular components. The accumulation of oxidative damage within a cell over the years has been proposed to be the main driving force in the ageing process (Harman, 1956). However, free radical theory was later refined due to discovery of superoxide dismutase in isolated mitochondria. Since the mitochondria are the major site within the cell for production of reactive oxygen species (ROS) and free radicals the theory evolved to a mitochondrial theory of ageing (Harman, 1972).

According to the mitochondrial theory of ageing, free radicals damage mtDNA and causes mtDNA mutations to accumulate during ageing. During normal aerobic respiration mitochondrial ROS are normally generated at very low levels and removed by antioxidants or by free radical scavenging enzymes. However, in ageing tissues, ROS production is increased and capabilities of scavenging enzymes are decreased. Increased ROS level could cause oxidative damage to different cellular components, but also to mtDNA, causing somatic mtDNA mutations. Accumulation of somatic mtDNA mutations could further lead to the ETC dysfunction that would foster ROS formation and mtDNA mutation accumulation. This vicious cycle would eventually cause a progressive decline in cellular energy conversion and an overproduction of ROS. The mitochondrial theory of ageing thus predicts that mtDNA damage exponentially increases over time and ultimately leads to ageing, degeneration and cell death. It was proposed that mtDNA is highly susceptible to oxidative damage due to its close proximity to the ETC. Damage to mtDNA caused by oxidative damage varies from base modification (8-oxo-2'-deoxyguanosine), formation of abasic sites to DNA strand breaks. The first report on mtDNA damage involvement in ageing comes from a study on the integrity of mtDNA in young adult and senescent rats. Using electron microscopy of reconstituted mtDNA duplexes, this study reported an increased abundance of structural aberrations consistent with mtDNA deletions in aged animals (Piko et al., 1988). In addition to mtDNA, ROS

can cause oxidative damage to RNA, proteins and lipids in the cells (Wei et al., 2009). Thus, it also affects ETC complex subunits. Some protein changes that occur during aging are specific for certain respiratory subunits (Choksi et al., 2007, 2008). However, it remains unknown whether these modifications would affect complex activity. A mouse model termed "mtDNA mutator" with defective proofreading activity of the POLG, provided the first causative link between mtDNA mutations and mammalian ageing (Trifunovic et al., 2004). These mice developed three- to fivefold increase in mtDNA point mutations as well as increased amounts of deleted mtDNA molecules. mtDNA mutations in this model accumulated in a rather linear fashion during life, thus opposite to the predictions of the theory (Trifunovic et al., 2005). The mtDNA mutator mice do not show any obvious phenotype at birth and in early adolescence, but subsequently acquire features of premature ageing such as weight loss, reduced subcutaneous fat, alopecia, kyphosis, osteoporosis, anemia, reduced fertility, heart disease, sarcopenia, progressive hearing loss and decreased spontaneous activity (Trifunovic et al., 2004). This model confirms that mtDNA point mutations can cause ageing phenotypes if present at high enough levels. However, it does not prove that the levels present in normal ageing are sufficient to cause ageing phenotypes. Despite the clear increase in the somatic mtDNA mutations in mtDNA mutator mice, no increase in ROS production was observed, suggesting that respiratory chain dysfunction *per se* is the primary inducer of premature ageing in mtDNA mutator mice (Trifunovic et al., 2005).

Most evidence supporting the free radical theory originates in comparative biology and intervention studies (López-Torres et al., 2002; Sohal et al., 1990). The free radical theory was tested in various models by modification of antioxidative defense system. However, a large amount of data coming from these studies does not result in a conclusive answer. Most of the studies that speak in favor of free radical theory were conducted in *Drosophila*. One of the best evidences for the free radical theory of ageing comes from the study showing that combined overexpression of *sod1* and *sod2* increases *Drosophila*'s lifespan proportionally to the level of SOD overexpression (Sun et al., 2004). However, overexpression of only *sod1* gene in *Drosophila* resulted either in increase, decrease or no effect on the lifespan at all, whereas their complete removal induces the lethality. These data point on a critical role of SODs in the cell, but it does not prove that superoxide radicals cause ageing in normal conditions. The three basic predictions of free radical theory of ageing – (i)

long lived individuals should have less ROS than short lived ones, (ii) decrease in ROS should increase the lifespan and (iii) increase in ROS should decrease the lifespan - were tested in the recent *Drosophila* study (Sanz et al., 2010). The first hypothesis was tested in three wild isolates of fruit flies, which showed that the longest-lived strain had the lowest ROS level, speaking in favor of the free radical theory. The second hypothesis was tested by expression of an alternative oxidase (AOX) from the urochordate *Ciona intestinalis* in fruit fly. AOX is localized in the IMM in plants, some fungi, protists and lower metazoa. It accepts electrons from coenzyme Q and by bypassing CIII and CIV transfer them directly to oxygen thus lowering ROS production. AOX expression in the fruit fly decreased ROS production, but did not increase lifespan, contradicting the theory. The third hypothesis was tested in aged dj-1β fly mutants known to have increased ROS that leads to a severe impairment of locomotive function as they age. However, the lifespan of these flies was not decreased, once more opposing the free radical theory (Sanz et al., 2010). Taken together, these results suggest that decreased mitochondrial ROS does not necessarily cause lifespan extension in flies.

Similarly, in mice complete removal of the MnSOD induced cardiomyopathy and neuordegeneration leading to early postnatal death. Knockout mice for cytosolic Cu/ZnSOD had severe oxidative damage and shortened the lifespan (Elchuri et al., 2005). However, deficiency of murine extracellular superoxide dismutase does not decrease lifespan and overexpresion of antioxidant defense in mice does not generally increase lifespan (Carlsson et al., 1995; Perez et al., 2009). Moreover, according to the free radical theory decrease in the function of MnSOD should affect life span, but this was shown not to be a case. *Sod2*$^{+/-}$ mice that had only 50% of MnSOD activity exhibit the significant increase in the level of oxidative DNA damage, but their life span was indistinguishable from the wild type. In addition, these mice did not present any difference in the aging biomarkers when compared to wild type (Van Remmen et al., 2003). Studies from mouse models imply that oxidative damage does shorten the life span, but its implication in the ageing process per se is still highly debated.

Observations in *C. elegans* do not match the data from mice, yeast and flies. *sod-2 C. elegans* deletion mutants showed a prolonged lifespan despite a significant increase in oxidative damage, while *sod-1* deficient mutants did not have any significant change in their lifespan. Most strikingly, *sod-1; sod-2* double mutants showed prolonged lifespan besides having the highest sensitivity to oxidative stress

(Van Raamsdonk and Hekimi, 2009).

Until now only a few experimental models could be interpreted in favor of the free radical theory of ageing while many still remain inconclusive (Muller et al., 2007). Consequently, the mitochondrial theory of ageing still remains debated and further data are needed to understand the role of oxidative damage in determination of the animal lifespan.

### *1.8.2. The Longevity Pathways*

Average lifespan has increased over the last centuries, due to substantial improvements in medicine and environmental factors, but maximal lifespan not as much. Many types of interventions, including genetic manipulation and dietary restriction, have been shown to increase the maximal life span in different animals models. Ageing studies in mammals are challenging due to their long lifespan and limited set of genetic tools that are crucial for assessment of the genome wide changes that occur during ageing. In contrast to mammals, *C. elegans* has been the model of choice for ageing studies during the past decades. It has relatively simple development. The embryonic development of *C. elegans* lasts approximately 14 hours (Sulston et al., 1983). After hatching, the worm proceeds through four molts to adulthood. Similarly to mammals, ageing nematodes exhibit a decline in mobility, chemotaxis, and reproductive capacity. The short lifespan, body transparency, simple cultivation, powerful genetic toolkit and the known location and fate of its 959 post-mitotic cells make *C. elegans* an excellent model to study age-related changes and ageing process *per se*.

#### *1.8.2.1. Insulin/IGF-1 Signaling*

The insulin/IGF-1 (IGF-1: insulin-like growth factor 1) signaling pathway was one of the first shown to influence ageing in a broad range of species (nematodes, flies and rodents). Suppression of this pathway increases the lifespan and delay age-dependent functional decline, while its up-regulation shortens the lifespan (Tatar et al., 2003). Ageing research was drastically promoted by the isolation of *C. elegans age-1* and *daf-2* mutants that live two times longer than wild type worms (Friedman

& Johnson, 1988; Kenyon et al., 1993). DAF-2 is the sole insulin/IGF-1 receptor in the worm, and AGE-1 corresponds to phosphoinositide-3-kinase (PI3K). Beside the exceptionally long lifespan, these mutants showed high resistance to oxidative stress, hypoxia, heat stress and heavy metals (Kaletsky & Murphy, 2010). DAF-2 signals through a serial cascade of kinases, resulting in phosphorylation of the Forkhead Box O (FOXO) transcription factor, DAF-16. The phosphorylation results in binding of the 14-3-3 chaperone that leads to nuclear exclusion of DAF-16 (Biggs et al., 1999). Without activation of DAF-2, DAF-16 gets translocated to the nucleus, promotes transcription of the downstream targets that would further induce diapause, stress resistance and longevity. Downstream targets of DAF-16 are stress-response genes (catalases, SOD, S-transferases and methalathioneins), chaperones, apoliproteins and lipases. Changes in the insulin/IGF-1 (IIs) pathway in one tissue lead to the changes in others. Environmental cues are sensed likely by neurons that inactivate DAF-2 and activate DAF-16. Hormonal signals are then sent to other tissues, decreasing IIs (through DAF-2 inactivation) and activating DAF-16 in these tissues (Wolkow et al., 2000). Overall, DAF-16 leads to longevity by simultaneously governing a magnitude of processes. Similarly, mutation in the DAF-2 homologue from *Drosophila* (InR) and mouse (IGF-1R, heterozygous) increased longevity (Holzenberger et al., 2003; Tatar et al., 2001). Perturbation of insulin/IGF activity seems to increase lifespan in humans, too. Mutations known to impair IGF-1 receptor function are overrepresented within the Ashkenazi Jewish population of centenarians (Suh et al., 2008).

#### *1.8.2.2. Germline Signaling Pathway*

Reproductive tissues have been shown to influence the life span of *C. elegans*, fruit fly, mice and humans (Kenyon, 2010a). DAF-16 strongly influences *C. elegans* longevity via regulation of the reproductive state independently of IIS signaling. Laser ablation of the germline primordial cells dramatically extends lifespan, whereas removal of the entire reproductive system does not have such an effect (Arantes-Oliveira, 2002; Hsin & Kenyon, 1999). This suggests that the germline and the somatic reproductive tissues have opposite effects on lifespan, with the germ cells preventing and the somatic gonad promoting lifespan extension. Nuclear localization of DAF-16 in adult intestinal cells is observed in germline ablated animals only. Thus, intestinal cells are likely the targets of reproductive signals (Berman &

Kenyon, 2006). The reproductive pathway acts in parallel to IIs, as loss of germ line almost doubles the lifespan of *daf-2* mutants (Hsin & Kenyon, 1999).

#### 1.8.2.3. Dietary Restriction

Dietary restriction (DR) is the only non-genetic intervention that increases lifespan in majority of species and was recently shown to improve health-span and reduce deaths due to age-related changes in the rhesus monkeys (Colman et al., 2009; Masoro, 2005). A number of genes mediating DR-induced longevity have been identified in *C. elegans*. Several different methods for DR-induced longevity have been described in *C. elegans*, ranging from genetic mutation that reduce food intake due to a defect in pharyngeal pumping (*eat-2)* to dilution of bacterial food or complete removal of bacterial food during adulthood. Intriguingly, different methods of dietary restriction extend *C. elegans* life span via mostly independent, partially overlapping pathways (Greer & Brunet, 2009). This is likely due to the fact that some nutrients are more limiting than others in different DR methods.

The role of Sir2 family of NAD dependent deacetylases - collectively known as sirtuins – in longevity induced by dietary restriction came first from studies in yeast (Lin et al., 2000). Yeast SIR2 mutants did not have increased replicative life span (measured by the number of budding events) when cultured in media with reduced glucose concentration (Lin et al., 2000). Overexpression of SIR2 homologues also extends the life span in yeast and *Drosophila* (Kaeberlein et al., 1999; Rogina & Helfand, 2004). However, other studies challenged these findings and suggest that the Sirt2 might play a role in DR but not as a master regulator, (reviewed in (Mair & Dillin, 2008)). The role of sirtuins in the longevity upon dietary restriction in *C. elegans* is also debated. Some studies support the role of *SIR-2.1* (SIR2 homologue), while others dismiss it (Hansen et al., 2007; Tissenbaum & Guarente, 2001; Wang & Tissenbaum, 2006). One possible explanation for this discrepancy in the conducted studies could be explained by the different dietary regimes that were applied. This could likely lead to a different requirement for Sir-2.1. In mammals, the role of sirtuins in the CR is not still completely understood and is currently an area of great interest.

Another signaling pathway that was found to regulate DR induced longevity in yeast, worms, and flies and recently in mice is the TOR (target of rapamycin)

pathway (Harrison et al., 2009; Selman et al., 2009; Stanfel et al., 2009). The TOR kinase regulates cell growth and metabolism in response to environmental cues. It couples growth factors and nutrients to protein homeostasis. TOR promotes protein synthesis by activating S6 kinase and inhibiting translation initiation binding protein (4E-BP) (Chen et al., 2009). TOR kinase regulates protein homeostasis, stimulates growth and blocks salvage pathways (e.g. autophagy) when enough food is available. *C. elegans* mutants for S6 kinase are long-lived and this longevity requires AMP activated kinase (AMPK) activity. Similar findings had been observed in mice (Selman et al., 2009). In worms, the PHA-4 transcription factor is also required to extend adult life span in response to reduced TOR signaling. *pha-4* encodes a FoxA transcription factor involved in mammalian embryonic foregut development as well as in control of postpartum growth and longevity. When food is plentiful, nutrients promote TOR and S6 kinase signaling, which represses *pha-4*/FoxA, leading to shorter lifespan (Sheaffer et al., 2008). However, PHA-4 is exceptionally required just in some form of DR in *C. elegans*. Reduction of PHA-4 does not suppress the long lifespan of *daf-2* mutant animals or in animals with impaired ETC function (Panowski et al., 2007).

DR in *C. elegans* extends lifespan generally independently from DAF-16. However one exception was observed where AMPK and DAF-16 mediates the effects on the life span of two particular form of DR in *C. elegans* (Greer et al., 2007).

In summary, there are plenty of genes that account for DR-induced longevity. However, it remains to be determined how and if they are unified in one common pathway.

#### *1.8.2.4. Mitochondrial Longevity Pathways*

While severe mitochondrial dysfunction often results in harsh pathologies, mild inhibition of respiration can lead to life span extension in worms, flies and mice (Copeland et al., 2009; Lapointe & Hekimi, 2008; Lee et al., 2003; Rea et al., 2007). A partial disruption in components of all ETC complexes (except CII) as well as of assembly factors can lengthen worm lifespan from 20% to 200% (Lee et al., 2003; Rea et al., 2007). These long-lived mutants, including those defined by RNA interference (RNAi), have collectively been termed “Mit mutants” (Rea, 2005). The level of ETC inhibition matters, and while low-level inhibition does not influence life

span, mild inhibition leads to life span extension. Severely affected ETC function in null-mutants tends to shorten life span (Rea et al., 2007). This suggests that there is a certain threshold of ETC dysfunction above which life span cannot be prolonged anymore. Moreover, a decrease in respiration during development, before L3/L4 transition, is sufficient to prolong the adult life span. The lifespan increase in Mit mutants is usually followed by changes in the larval development, fertility and adult size (Dillin et al., 2002; Rea et al., 2007). Beside the Mit mutants, who have loss of function or reduced activity of ETC components, other mutations that affect substrate availability to ETC, e.g. transporters of TCA cycle substrates or pyruvate dehydrogenase, also lead to life span extension (Rea, 2005). Mit mutant longevity occurs independently of the IIs pathway and germline signaling (Butler et al., 2010; Dillin et al., 2002).

#### *1.8.2.5. The Mit Mutants*

Only a small number of Mit mutants have been identified genetically (Lee et al., 2003; Rea, 2005) (Table 1).

| Strain | Gene affected | Life span |
|---|---|---|
| *gas-1(fc21)* | 49kDa subunit of CI | short-lived |
| *nuo-6(qm200)* | NDUFB4/B15 subunit of CI | long-lived |
| *clk-1(e2519)* | demetoxyubiquinone hydroxylase (ubiquinone synthesis) | long-lived |
| *mev-1(kn1)* | cytochrome b subunit of CII (SDHC) | short-lived |
| *isp-1(qm150)* | Rieske FeS protein of CIII | long-lived |
| *isp-1(qm150), ctb-1(qm189)* | Rieske FeS protein CIII cytochrome b of CIII | long-lived |
| *ucr-2.3(pk732)* | core protein of CIII | short-lived |

**Table 1. Genetically defined Mit Mutants.**

Several hypotheses have been proposed in order to explain mitochondria-induced longevity, yet a clear answer has not been found.

I. Reduced metabolic rate, resulting from slow embryonic development, larval growth and behavior, could lead to decreased ROS production and thus slower ageing

(Rea, 2005).

II. Mild increase in ROS formation in Mit mutants due to ETC dysfunction might act as 'hormetic' signal that leads to activation of the antioxidant defense system (Rea, 2005). However, most of the long-lived genetic Mit mutants do have elevated expression levels of *sod-1* and *sod-2*, likely due to an increased level of superoxide radicals, but these elevations are not essential for their longevity (Yang et al., 2007). Indeed the increased generation of superoxide radicals in *isp-1(qm150)* and *nuo-6(qm200)* mutants is required to regulate longevity, most likely at the level of gene expression. Consequently, the antioxidant n-acetylcysteine (NAC) drastically decreased the life span of these mutants, while it did not have any effect on wild type animals (Yang & Hekimi, 2010a). Consistent with this, mild treatment with paraquat and juglone, chemical compounds known to induce oxidative stress, extends life span of wild type animals (Hwang & Lee, 2011; Lee et al., 2010). It was observed that elevated ROS was responsible for HIF-1 (hypoxia inducible transcription factor) activation, which further regulates the immune response in *mClk-1*$^{+/-}$ mice under normal oxygen condition (Wang et al., 2010). HIF-1 is a heterodimer of HIF-1α and HIF-1β subunit (Semenza, 1995). Both subunits are ubiquitously expressed, but their stability is differentially regulated by oxygen levels. HIF-1α is rapidly degraded in normoxia and present only in hypoxia, whereas HIF-1β protein levels are stable and not affected by oxygen level. During normoxia HIF-1α is targeted for degradation by hydroxylation via prolyl hydroxylases (PHDs), which require Fe(II) bound to the catalytic center, oxygen and 2-oxoglutarate as a substrate. Upon hydroxylation, interaction with von Hippel-Lindau tumor suppressor protein (pVHL) will enable degradation of HIF-1 by 26S proteasome-dependent degradation. Hypoxia prevents hydroxylation of HIF-1α leading to protein stabilization and its accumulation in the nucleus (Wheaton & Chandel, 2011). In *C. elegans* the link between ROS, HIF-1 and longevity of Mit mutants derived from the observation that HIF-1 was necessary for the extended life span of *clk-1* and *isp-1* mutants under normal oxygen conditions without affecting brood size, behavior, and reproduction. This study has suggested that impaired respiration observed in long-lived Mit mutants could further lead to activation of HIF-1 that would then regulate gene expression to trigger longevity (Lee et al., 2010). These observations beg a question for the role of HIF-1 in short-lived Mit mutants that also have increase in ROS? Still, it is unclear how ROS change HIF-1 activity and what are the HIF-1 target genes in Mit mutants. It has been observed in

mice that ISP protein of CIII might play a major role in oxygen sensing within the cell through a ROS-dependent mechanism, whereas oxidative phosphorylation and mitochondrial respiration do not influence HIF-1α stabilization under hypoxic conditions (Brunelle et al., 2005). Superoxide radicals derived from the ISP protein could convert further to hydrogen peroxide in the cytosol where they could oxidize PDH's Fe cofactor thus making it catalytically inactive and in turn stabilize HIF-1 (Wheaton & Chandel, 2011). However, the role of ROS derived from CIII in HIF-1α stabilization in hypoxia was recently questioned (Chua et al., 2010). Furthermore, while some studies showed that HIF-1 stabilization extends *C. elegans* lifespan, others show that loss of HIF-1 under normoxic conditions can either prolong or not have any affect on the life span of the wild type animals (reviewed in (Leiser & Kaeberlein, 2010). Recent study tried to reconcile these observations by proposing that stabilization of HIF-1 promotes longevity by a mechanism distinct from IIS and DR, but can also negatively regulate longevity by repressing nuclear localization and activation of DAF-16 in a temperature dependent manner (Leiser et al., 2011). In summary, the role of ROS, respiration and oxidative phosphorylation in hypoxia-induced longevity remains highly debatable.

Another example of mitochondrial hormesis was observed in animals that were fed with a glucose-restricted diet that led to increased ROS and mitochondrial respiration. However, these animals had extended life span and an increase in oxidative stress resistance (Schulz et al., 2007). These observations suggest that ROS, produced due to ETC dysfunction, could activate a mitochondrial retrograde signaling pathway that would, in turn, promote a hormetic effect. The idea behind this hypothesis is that early exposure to mitochondrial ROS might enhance oxidative stress resistance later in life (Schulz et al., 2007; Woo & Shadel, 2011).

III. The presence of rhodoquinone and fumarate reductase homologue in *C. elegans* led to another hypothesis. Fumarate reductase (FR) is coupled to rhodoquinone, which permits efficient fumarate reduction, and might bypass CIII and CIV of the ETC. FR might generate high amounts of ROS, as seen in bacteria, that would consequently induce an antioxidant response that hormetically increases life span (Rea, 2005). In anaerobic conditions, a fermentation process takes place in order to generate ATP and maintain redox balance. In *C. elegans*, malate dismutation is one of such processes, which catalyzes the NADH/$FADH_2$-dependent reduction of fumarate to succinate. *C. elegans* has two identified FRs: the cytosolic or soluble FR,

sFR (F48E8.3), and mitochondrial FR, SDHA-1 (a subunit of CII, C03G5.1) (Rea & Johnson, 2003). Surprisingly, RNAi against sFR further increased life span of the long-lived mutants *isp-1(qm150)*, *clk-1(qm30)* and *clk-1(e2519)*, but did not change the life span of the short-lived mutants *mev-1(kn1)*, and *ucr-2.3(pk732)*. In addition, wild type animals showed a significant life span decrease upon RNAi depletion of sFR (Butler et al., 2010). This data indicate that sFR probably acts in parallel to the pathways underlying mitochondria-dysfunction induced longevity.

IV. Mit mutants might utilize an alternate ATP production pathway that does not require oxygen as a terminal electron acceptor and this metabolism may lead to the longevity. Metabolic profiling of two long-lived mutants indicated on the possible distinction between short and long-live Mit mutants in their metabolic products. While long-lived *clk-1(qm30)* and *isp-1(qm150)* mutants showed a two fold increase in pyruvate, two short-lived mutants, *mev-1(kn1)* and *ucr-2.3(pk732)*, had higher levels of metabolites including lactate, α-ketoglutarate and dipeptide Gly-Pro (Butler et al., 2010). Transcriptome analysis of *clk-1(qm30)* mutants identified an up-regulation of genes encoding enzymes involved in oxidative phosphorylation, glycolysis, gluconeogenesis, amino-acid metabolism, fatty acid and nucleotide metabolism, cellular detoxification system, xenobiotic metabolism and anaplerotic pathways. However, these changes were also observed in short-lived *gas-1* mutant animals, probably as a result of mitochondrial dysfunction (Cristina et al., 2009; Falk et al., 2008). Transcriptome analysis of *isp-1* mutants identified up-regulation of the detoxification response in addition to anaplerotic pathways. Mutation in *cdr-2*, a member of the gluthatione S-transferase family involved in cellular detoxification response, drastically reduces the long life span of *isp-1* and likely *clk-1* mutants (Cristina et al., 2009). However, the effect of these genes on short-lived Mit mutants was not examined.

Other genes have also been identified as regulators of Mit mutant longevity. AMPK (AAK-2) partially regulates the longevity of *clk-1* and *isp-1* mutants. However, AAK-2 depletion further enhances the feeding and growth defects in these mutants (Curtis et al., 2006). Mutations in *fstr-1* and *aqp-1* specifically altered longevity in *clk-1* mutants but not in wild type. *aqp-1*, encodes a glycerol channel that also contributes to the long life span of *daf-2* mutants (IIs pathway). The exact function of FSTR-1 is still unknown. *fstr-1* gene is expressed in a nerve ring interneuron, two pharyngeal interneurons and the intestine. RNAi against *fstr-1*

improved the rate of development in *clk-1* mutants and enhanced pumping and trashing rates, but slowed both parameters down in wild type animals. *fstr-1/2* RNAi did not affect the lifespan of *isp-1* mutants. On the contrary, it further lowered the developmental rate, leading to developmental arrest in many animals (Cristina et al., 2009). This might suggest that the longevity phenotypes in *isp-1* and *clk-1* mutants are due to different mechanisms.

So far, only one gene, the homeobox transcription factor *ceh-23*, seems to specifically regulate life span of long-lived genetic Mit mutants (*clk-1*, *isp-1* and *isp-1;ctb-1*) while not having any effect on mean life span of wild type animals or short-lived Mit mutants (*mev-1*). Similarly to *fstr-1*, *ceh-23* expression was observed in the intestine and in a set of neurons. In contrast to the *fstr-1*, *ceh-23* extends the life span of the long-lived *clk-1*, *isp-1* and *isp-1;ctb-1* mutants without affecting developmental rates and fertility, suggesting that *fstr-1/2* and *ceh-23* might act through independent pathways. Overexpression of *ceh-23* in *isp-1*;*ctb-1* mutants extends their life span even further without affecting developmental rate (Walter et al., 2011).

Intriguingly, tissue specific knockdown of mitochondrial ETC subunits in either neurons or intestinal cells, but not in body muscle cells, is sufficient to extend lifespan. Moreover, tissue specific ETC knockdown uncouples developmental defects such as increased resistance to oxidative stress, decreased body size, slow development, and reduced fertility, from longevity (Durieux et al., 2011). Mitochondria possess protein machinery responsible for proper protein folding and complex assembly, termed mitochondrial unfolded protein response, $UPR^{mt}$. Matrix-localized chaperons, HSP-6 and HSP-60, are required for protein import and facilitate protein folding, whereas proteases (e.g. ClpP) localized in the matrix and IMM degrade the protein that fail to fold or assemble correctly (Haynes & Ron, 2010). Disruption of ETC complexes either by RNAi or genetic mutations activates $UPR^{mt}$ (Durieux et al., 2011; Yoneda et al., 2004). ETC inhibition must occur before L4 larval stage in order to activate $UPR^{mt}$, thus matching the timing requirements of the life span extension for ETC RNAi-defined mutants (Durieux et al., 2011). Down-regulation of ETC subunits in neurons activates $UPR^{mt}$ in the intestine, suggesting that an still unknown signaling molecule released from the nervous system can induce $UPR^{mt}$ in the intestine. This suggests that $UPR^{mt}$ can be activated in a non-autonomous manner (Durieux et al., 2011; Woo & Shadel, 2011). However, ETC impairment in the body wall muscle also activates $UPR^{mt}$ in the intestine but does not

affect life span. In addition, up-regulation of UPR was seen also in short-lived Mit mutant (*mev-1(kn1)*) suggesting that $UPR^{mt}$ could also be uncoupled from the longevity.

The recent findings described above present just a few pieces of a big puzzle that remains to be resolved. A better understanding how its parts are connected and how or if they function together awaits further investigations. Elucidating the mechanisms that link mitochondria and longevity may ultimately lead to preventive measures or even therapies for age-related diseases and thus contribute to healthier lives in humans.

# 2. Aims of this Study

Mitochondria have an important role in the life of eukaryotic cells by controlling their metabolic rate, energy production and the cell death. Thus, they are involved in the ageing processes, too. The aim of this work presented here was to study the role of mitochondria and mitochondrial dysfunction in ageing by using *C. elegans* as a model organism.

**The specific aims were to:**

**Paper I**: develop methods to study mitochondrial DNA levels and the role of mtDNA maintenance during the development of *C. elegans*

**Paper II**: study the regulation of mtDNA replication during early development of *C. elegans*. To establish the timing and importance of active mtDNA replication in maintenance of somatic tissues Vs. germline in *C.elegans*

**Paper III:** develop methods that will allow analysis of morphological and metabolic changes on a level of single tissues in *C. elegans*

**Paper IV**: determine the role of succinate dehydrogenase as an essential metabolic regulator of longevity in *C. elegans* mitochondrial mutants

# 3. Results and Discussion

## 3.1. mtDNA Copy Number is the Limiting Factor for *C. elegans* Development and Differently Affects *C. elegans* Tissues (Paper I, II)

*C. elegans* mtDNA is a double stranded, circular DNA molecule of 13794 bp in length. It encodes for 2 ribosomal rRNAs (12S and 16S rRNA), 22 tRNAs and 12 subunits of mitochondrial respiratory chain complexes (Figure 1). In contrast to the majority of metazoan species, it lacks the ATP8 gene that encodes for one of CV subunits. The *C. elegans* mtDNA has two non-coding regions: the D-loop, AT rich, 466 bp long, located between $tRNA^{ala}$ and $tRNA^{pro}$, and a 109 bp long non-coding sequence located between the *ND4* and *Cox-1* genes (Okimoto et al., 1992). The precise role of these non-coding regions is still unknown. Based on their arrangement, in *C. elegans* all mtDNA genes are likely transcribed in the same direction, from the heavy strand only. The 12S and 16S rRNA genes are separated by protein encoding genes, while they are adjacent in mice and *Drosophila*. This suggests that different mechanisms of transcription regulation might exist in *C. elegans*. So far, very little is known about the transcription and replication mechanisms in nematodes, mainly due to lack of experimental approaches to study these processes. Even though tremendous effort has been put into elucidating mtDNA replication, the mechanism that determines the level of mtDNA in a single cell still remains unknown.

To assess the role of mtDNA copy numbers during the *C. elegans* life cycle, we developed several new methods (Paper I). We accurately determined the mtDNA copy number in single animal relative to dilutions of a plasmid containing a fragment of NADH dehydrogeanse subunit 1 (*nd-1*). In order to identify the exact moment in development when mtDNA replication starts, we followed the amount of mtDNA relative to nuclear genome content using actin (*act-3*) as the reference gene. We found that embryos and early larvae, until L3 stage, contain about 100.000 mtDNA molecules. This suggests that mtDNA replication is not initiated until L3. These results correlate with the finding in mice that mtDNA replication is paused during the pre-implantation period of an embryo (Cao et al., 2007, 2009; Cree et al., 2008; Wai et al., 2010). Further analysis showed that mtDNA copy number gradually increases to approximately 4.000.000 in young adults. This burst in replication matches the onset of germline proliferation, suggesting that the majority of mtDNA is synthesized

in the gonad (Paper I).

mtDNA is replicated by the mitochondrial DNA polymerase, POLG. The holoenzyme structure of POLG varies among different organisms. In *S. cerevisiae* it consists of catalytic subunits only, while there is one additional accessory subunit in *Drosophila* and they are two in mammals (Kaguni, 2004; Lee et al., 2009; Viikov et al., 2011). The importance of POLG during development and mitochondrial function has been shown in model systems ranging from yeast to mammals. In yeast, mutation in the catalytic subunit of POLG, encoded by the *mip-1* gene, results in mtDNA depletion and formation of $rho^0$ cells that cannot perform OXPHOS (Genga et al., 1986). Flies deficient in POLG activity, *tam* mutants, die before pupa stage (Iyengar et al., 1999, 2002). In mice, POLG deficiency causes embryonic lethality during late gastrulation, even before organogenesis (Hance et al., 2005). In *Drosophila*, the level of POLG-B follows mtDNA copy number, while over-expression of the catalytic subunit alone does not increase mtDNA copy number (Lefai et al., 2000). The same was found in human cell lines (HEK293T), and the expression level of the catalytic subunit is rather constant in all tissues (Schultz et al., 1998; Spelbrink et al., 2000). mtDNA copy number in mammals is specific for tissue types and developmental stages (Clay Montier et al., 2009).

In *C. elegans*, the gene Y57A10A.15, now termed *polg-1*, shows 26% identity and 45% similarity to the human POLG-A catalytic subunit. Multiple sequence alignment revealed the presence of highly preserved motifs in the polymerase (A, B and C) and exonuclease domains (Exo I, Exo II, Exo III). Orthologs to the mammalian POLG-B accessory subunits have not been found in *C. elegans* (Lucas et al., 2004).

We performed phenotypic characterization of the two deletion alleles *polg-1(tm2685)* and *polg-1(ok1548)* (Paper I and II, respectively). *ok1548* is a 2149 bp deletion that removes exons 8 to 10 of the predicted POLG polymerase domain, while *tm2685* is a 486 bp deletion that removes part of the two first exons of the predicted exonuclease domain. Both *polg-1* mutants contain drastically decreased mtDNA levels in adulthood. mtDNA depletion mostly leads to mitochondrial dysfunction in mammalian tissues with high energy demands. Strikingly, homozygous *polg-1* mutant animals had a normal development despite mtDNA depletion. This was a rather surprising finding considering that the lack of POLG leads to lethality in flies and mice (Hance et al., 2005; Iyengar et al., 1999). We explained this seemingly

paradoxical phenomenon by high maternal contribution which would account for most somatic, yet not germline mtDNA. Still, both *polg-1* mutants had a shortened life span caused by fatal intestinal protrusion through the vulva, occurring in nearly all individuals (Paper I, II). In order to examine changes in post-mitotic tissues of *polg-1* animals we adjusted established methods for ultrastructural and *in vivo* analysis of mitochondrial morphology (Paper I). Specifically, transmission electron microscopy and a muscle-mitochondria-targeted GFP in homozygous *polg-1(ok1548)* revealed that mitochondria were more disorganized and formed larger units compared to wild type. This increase in fusion or decrease in fission could indicate a strategy for functional complementation of mtDNA-encoded proteins. Mitochondria in hypodermal cells, intestine and body wall muscles of *polg-1* deficient animals appeared to be unchanged on the ultrastructural level (Paper II).

To elucidate functional consequences of mtDNA depletion, we examined a physiological rhythm of the soma (Paper II). Defecation behavior in worms involves a small number of muscles, a set of neurons, and the neurotransmitter GABA (Branicky & Hekimi, 2006). Each defecation cycle in *C. elegans* starts with a slow posterior body wall muscle contraction (pBoc), and is followed about a second later by relaxation. About 2 seconds later, a sharp contraction in the head of the animal (aBoc, anterior body contraction) occurs, anal muscles rapidly contract and slurry feces appear (Exp, expulsion phase) (Liu & Thomas, 1994; Hart, 2006; McGhee, 2007). We detected a progressive increase in pBoc and Exp interval length with age of wild type animals while defecation rates in *polg-1* got much shorter at adult day 6. These findings indicate that somatic tissues of homozygous *polg-1* mutants are not severely affected by mtDNA depletion (Paper II).

mtRNA levels were shown to be proportional to mtDNA copy numbers (Annex & Williams, 1990). The mitochondrial genome with its lack of introns and specific organization presents a challenge to accurate estimation of transcript levels. Discrimination between transcripts and mtDNA is problematic, since their sequences are identical. We improved the accuracy of established methods by introducing a set of controls that monitor potential DNA contamination in RNA samples. Even though DNAse treatment is quite effective, we have demonstrated that failure is possible and that it drastically influences the results. In order to detect mtDNA contamination in RNA samples, we designed two primer pairs, one spanning the D-loop region and another spanning the $tRNA^{Pro}$ and $tRNA^{Val}$ genes (Paper I). We observed that

transcript levels for *ND5* and cytochrome b were significantly decreased in *polg-1* animals, correlating with mtDNA depletion.

Despite the absence of severe phenotypes in the soma, we observed pronounced morphological defects in the gonad of *polg-1* mutants. This effect of mtDNA depletion is expected, considering that the gonad is the only highly proliferative tissue in adult worms. The germline defects led to functional infertility in homozygotes. Gonad development was normal until the first embryos were produced. At the first day of adulthood, drastic changes in mitochondrial morphology and number were observed within the germline. Later in adulthood, at day six, gonads in *polg-1(ok1548)* were essentially depleted of mitochondria, most likely due to the production of a small number of inviable embryos and the inability to replicate mtDNA. Interestingly, these embryos arrested at different stages, mostly before reaching four cells. Some developed until comma and even fewer to 3-fold stage. Extremely rarely, the first embryo of a young adult hatched, but then arrested as a larva. These individual differences in embryonic development could be attributed to maternally inherited mtDNA levels. These first embryos produced probably received a higher number of mtDNA copies than all following ones. The copy numbers in arrested embryos were approximately 30 times lower than in wild type, suggesting a certain threshold below which development is impossible. Time-lapse microscopy was used to examine the rate of development in these arresting embryos. The developmental rate progressively slowed down between four-cell and comma stage, likely due to mtDNA dilution with each successive cell division. This could in turn lead to mitochondrial failure, culminating in arrest (Paper II).

In summary, our data indicate that somatic tissues of homozygous *polg-1* mutants are not severely affected by mtDNA depletion and that mtDNA replication does not take place during and is not necessary for worm embryonic and larval development. However, they do show that *C. elegans* development and reproduction radically depend on mtDNA levels.

### 3.2. Development of a New Method to Follow Tissue-Specific Metabolic Changes in *C. elegans* (Paper III)

The mammalian OXPHOS capacity as well as effects of ETC dysfunction vary among tissue types. Besides the general understanding that ETC disturbance strongly

influences longevity and ROS production in Mit mutants, little is known about tissue-specific changes in these worms. Metabolic changes have mostly been followed in whole animals, since the small body size of this nematode leads to number of technical limitations for tissue level assessments. We aimed to adapt a palette of qualitative and quantitative histochemical reactions for analysis of *C. elegans* metabolic state at tissue level.

In order to get a reference of cross-sectional, light microscopic morphology in *C. elegans* we adjusted conventional hematoxylin-eosin (H&E) staining for use with frozen sections. We found that H&E provides good longitudinal and transverse resolution with readily identifiable tissues based on their structural characteristics. Differential binding properties of hematoxylin and eosin to tissue components are the base of current pathomorphological diagnostics in human patients and likewise reveal pattern deviations from a reference state in worms. We included *polg-1* deficient animals in our test series since we had demonstrated (Paper II) that these animals exhibit severe morphological abnormalities in the gonad. Cross sections revealed two pronounced changes: accumulation of an eosinophilic mass in the pseudocoelomic space and absence of a properly formed rachis, as previously identified by both TEM and epifluorescent microscopy. This further prompted us to examine the nature of the eosinophilic mass in the pseudocoelomic space by staining lipids with Oil-Red-O and high molecular weight carbohydrates with PAS (Periodic Acid Schiff's) reagent. This amorphous mass did not stain with either method and thus has characteristics of a protein-containing fluid comparable to intraalveolar lung edema and ascites in humans. We proposed that this pseudocoelomic fluid accumulation could explain the lethal organ protrusion observed in *polg-1* animals. One possible mechanism might be that mtDNA depletion gradually leads to a malfunctioning excretory system. Yet, we cannot exclude the possibility that the fluid accumulation occurs due to gonad defects.

The major fat storage in *C. elegans* is represented by triacylglycerides (TAGs) in the lipid droplets and yolk. Lipid droplets are mainly found in the intestine and hypodermis. Recently, a wide range of methods for analysis of worm lipid content was developed. Most of them are based on the analysis of either worm homogenates or whole worm mounts. One widely accepted stain for fat deposits in *C. elegans* is Oil-Red-O. To date, it had only been used in whole mount specimens (O'Rourke et al., 2009). We aimed to adjust Oil-Red-O staining to readily visualize and quantify fat

storage in frozen sections at tissue level.

Synchronized hermaphrodites were examined at the day 1 of adulthood. The specificity of our method was tested by analyzing the lipid storage abnormal alleles *daf-2(e1370)* and *lpd-3(ok2138)*. Enzyme-based colorimetric assays, thin layer chromatography and whole mount Oil-Red-O stained worms served as external references. Color image segmentation of Oil-Red-O stained and hematoxylin counter-stained sections determined the overall lipid fraction in individual animals and tissues. Cumbersome imaging of three-dimensional whole-mounts was entirely bypassed by the use of sections. We detected high lipid levels in the intestine and the proximal germline of wild type, while they were low in the distal gonad. The proportion of fat stored within the largest tissues of the worm - intestine and gonad - remains unchanged irrespective of overall lipid content. While our histochemical approach was able to detect two times more fat in *daf-2(e1370)* and half the amount in *lpd-3(ok2138)* relative to wild type, colorimetric analysis failed to show this change in *lpd-3*. This is likely due to a limitation of colorimetry if the lower threshold is approached. For a fair comparison of whole mount stained worms to our native sections, we subjected fixed, permeabilized, and Oil-Red-O stained worms to sectioning. We found that morphology and resolution are far better preserved in frozen sections that were natively stained with Oil-Red-O and hematoxylin. Being well suited for tissue level analysis, our method still lacks sufficient resolution to address subcellular details of lipid stores.

In *C. elegans*, the effect of different mutations in ETC complexes on their activity has been analyzed by either respiration assessment of whole animals - *in vivo* - or enzyme activity measurements in uncoupled sub-mitochondrial particles *in vitro* (Braeckman, Houthoofd, Brys, et al., 2002; Braeckman, Houthoofd, De Vreese, et al., 2002; Senoo-Matsuda et al., 2001; Van Voorhies, 2002). We aimed to quantify activities of different ETC complexes at a single tissue level - *in situ* - by specific enzymatic staining reactions.

We quantified NADH, SDH, and COX activities in fresh, frozen *C. elegans* sections by determining the optical density (OD) upon staining (Jung et al., 2002). Control reactions were performed in order to exclude possible unspecific staining. The reaction kinetics were monitored by time-lapse recording during staining and plotting of the temporal OD change. Linear correlation between OD of the dye and enzymatic activity within detection range was observed, and reference incubation

time ranges were defined. Quantitative assessment of NADH, SDH, and COX activities in wild type animals showed remarkable differences between individual tissue types in analogy to mammalian tissues. We observed high activities in the pharynx, body wall muscle and in the apical portions of intestinal cells of wild type worms, while they were low in germline. Even within the same tissue, higher activity was seen in the head muscles versus the remaining body wall. A set of mitochondrial mutants was independently analyzed by histochemistry - *in situ* -, respirometry - *in vivo* -, and spectrophotometry of isolated sub-mitochondrial particles - *in vitro*.

CI *gas-1(fc21)* mutants have decreased respiration, and a decrease in SDH activity of CII, measured *in vivo* and *in vitro*. Staining showed a mild increase in NADH in the intestine but not body wall muscle or pharynx, and a significant increase in SDH and COX activities in all tissues. This might suggest an increase in respiratory chain protein content *in vivo*, reflecting an attempt to compensate for the CI defect. Since *gas-1(fc21)* is not a null allele, residual NADH activity, combined with up-regulation of gene expression might account for the seemingly paradox result. Alternatively, the indicator system used in our study might react with other potentially up-regulated NADH-oxidizing enzymes.

*mev-1(kn1)* is a point mutation in the SDHC anchoring subunit of CII. In agreement with the decrease in respiration, we observed a significant decrease in the CII activity in isolated submitochondrial particles. However, *in vitro* and *in situ* measurements showed increased SDH activity suggesting that even if CII activity is affected, SDH activity may persist. This could be explained by the fact that CII links succinate dehydrogenase activity (SDH) in the TCA cycle with the reduction of ubiquinone in ETC (Figure 3). Thus, while the oxidation of succinate and electron transfer to the flavin group in SDHA subunit may be normal, subsequent transfer to iron sulfur clusters in SDHB subunit and to ubiquinone might be impaired resulting in decreased CII activity.

*polg-1(ok1548)* mutants have decreased NADH activity, while SDH was increased in all tissues, likely reflecting a compensatory mechanism. COX activity was reduced in all tissues, mostly in the pharynx. This indicates that the effect of mtDNA depletion is tissue specific, as in human disease, where various POLG mutations lead to tissue specific changes in ETC complex activities (Schaller et al., 2011). Similarly, $POLG^{-/-}$ mouse embryos had drastically reduced COX and normal SDH activities (Hance et al., 2005). CII subunits are all nuclear encoded and thus not

affected by mtDNA depletion.

### 3.3. Succinate Dehydrogenase Divergently Regulates Longevity Upon Mitochondrial Dysfunction in *C. elegans* (Paper IV)

Severe dysfunction of ETC complexes usually results in developmental arrest, mostly at L3 larval stage, while the mild changes can even increase *C. elegans* lifespan. However, not every deficiency in ETC complexes leads to an increased life span (Rea et al., 2007). Mutation in the SDHB subunit in *Drosophila* causes early mortality and age-associated behavioral decay, whereas RNAi against other ETC complexes can extend lifespan in this model (Copeland et al., 2009; Walker et al., 2006). Similarly, none of the known mutations in CII or RNAi against CII subunits lead to increased longevity in *C. elegans*, indicating its vital role. Thus, we aimed to experimentally define the specific role of CII with respect to Mit mutant longevity.

The response of short-lived *gas-1(fc21)* and *mev-1(kn1)* as well as long-lived *isp-1(qm150), isp-1(qm150);ctb-1(qm189)*, and *clk-1(e2519)* mutants to pharmacological inhibition of ETC complexes was analyzed. Worms were grown on NGM (nematode growth media) containing inhibitors for either CI - rotenone; CII - malonate; CIII- antimycin A; or CIV - sodium azide. We observed that both, short- and long-lived mutants have an increased resistance to CIV inhibition, as previously shown (Butler et al., 2010). *isp-1;ctb-1* showed increased resistance to CI inhibition in comparison to *isp-1* alone, likely due to the presence of more stabile I-II-IV supercomplexes (Suthammarak et al., 2010). In mammals, the vast majority of CI appears to be present as supercomplexes (17% in CI-CIII and 63% in CI-CIII-CIV) where CIII and CIV might have a role in stabilizing CI (Acín-Pérez et al., 2004, 2008; Suthammarak et al., 2010, 2009). *clk-1(e2519)* mutants showed a severe response to inhibition of CI. This could be explained by the reduced CI activity in these mutants (Kayser et al., 2004). Thus, further CI inhibition could be lethal. While we did not find any specific phenotype that would characterize either group of short-lived or group of long-lived Mit mutants upon CI, CIII, and CIV inhibition, we observed that all mutants and wild type animals responded similarly to blockage of CII. CII consists of four subunits (SDHA-SDHD), the two peripheral ones (SDHA/*sdha-2* and SDHB/*sdhb-1*) act as succinate dehydrogenase of the TCA cycle, while two others (SDHC/*sdhc-1*, SDHD/*sdhd-1*) are anchoring subunits. In contrast to mammals, the nematodes *C.*

*elegans* and *Ascaris suum* possess an additional CII subunit termed SDHA-1, which is homologous to *E. coli* fumarate reductase (FR). FR, mainly found in bacteria and parasitic animals, catalyzes the reduction of fumarate to succinate under anaerobic conditions (Cecchini et al., 2002; Kita, 1988; Kuramochi et al., 1994; Rea & Johnson, 2003). The key difference between *E. coli* SDH and FR is the distribution of redox potentials among redox centers: SDH contains two high redox potential centers ([3Fe-4S] and heme b), while FR contains FAD and [2Fe-2S]. The presence of these redox centers favor either succinate reduction in case of SDH or fumarate reduction in case of FR. Due to weaker redox centers in FR, high ROS production is very likely. In order to avoid this potential source of damage, bacteria and parasites tend to switch to SDH in aerobic conditions, while FR is preferred for anaerobic metabolism (Hederstedt, 2003; Yankovskaya et al., 2003). The interplay between these two functions of CII is likely regulated via differential expression of the flavoprotein subunit of CII and by presence of both, rhodoquinone and ubiquinone in *C. elegans* mitochondria. It has been proposed that the longevity of Mit mutants depends on FR activity (Rea, 2005). We tested the effect of SDH and FR activity on longevity of Mit mutants by RNAi against all five CII subunits in short-lived *gas-1* and long-lived *isp-1;ctb-1* animals. Knockdown of *sdha-1*, but not *sdha-2*, significantly reduces the life span of wild type worms, indicating the importance of FR activity. This is not surprising, as *C. elegans* naturally inhabits rather hypoxic environments. In contrast, down-regulation of every single CII subunit alone significantly decreases longevity of *isp-1;ctb-1*. Short-lived *gas-1* animals reacted differently to CII RNAi: Down-regulation of *sdha-1* did not affect life span, but RNAi against *sdha-2* shortened it. This is in line with the lethal phenotype of *gas-1;mev-1* double mutants (Hartman et al., 2001). Strikingly, knockdown of *sdhb-1, sdhc-1*, and *sdhd-1* in *gas-1* significantly increased life span, suggesting CII as longevity suppressor in this mutant background. These data pointed out that deficiency in catalytic and anchoring subunits lead to opposite phenotypes in *gas-1*. The same holds true in human disease caused by equivalent mutations. Defects in SDHA lead to bioenergetic failure, while mutations in SDHB, SDHC or SDHD induce tumor formation (Guzy et al., 2008). In summary, longevity of Mit mutants depends on SDH and CII activity, whereas FR activity influences life span in wild type animals but only modestly long-lived *isp-1;ctb-1* mutants. Lastly, we showed that lifespan of the long-lived *isp-1;ctb-1* can be prolonged by RNAi against *cco-1* that encodes for the Vb/Cox4 subunit of CIV,

further indicating that SDH activity might be required for their longevity.

To test the relation between longevity and respiration, we subjected long-lived *isp-1;ctb-1* to RNAi against *sdha-2*, which decreased their life span or against *cco-1* which led to further life span prolongation. We measured respiration *in vivo* and found that both RNAis lead to further decrease in respiration despite their opposite effects on longevity. In addition, the progeny of animals subjected to *cco-1* RNAi died during embryogenesis. This is consistent the fact that with severe mtDNA depletion, presumably leading to energy deficiency, leads to embryonic arrest (Paper II). Our data also indicate that respiration is not necessarily coupled to longevity.

It was proposed that a still unidentified signal, initiated during L3/L4 larval stage of development, was necessary for mitochondrial dysfunction-dependent longevity (Dillin et al., 2002). This hypothesis prompted us to determine the developmental stage in which CII influences longevity in Mit mutants. We subjected wild type and *isp-1;ctb-1* to RNAi against individual CII subunits either until or after L4. As expected, CII RNAi affected wild type life span only if administered during larval development. In *isp-1;ctb-1* we saw the opposite: CII RNAi knockdown from L4 onward had a dramatic effect on lifespan. Knockdown of *sdha-2* had the strongest effect, while down-regulation of *sdha-1* and *sdhb-1* had only a minor effect. This suggests that SDH activity is important for metabolic reprogramming required for lifespan prolongation in *isp-1;ctb-1*. Our observation, that RNAi against *sdha-2* had the strongest effect on lifespan in the long-lived *isp-1;ctb-1,* contradicts the hypothesis that a shift towards malate fermentation through FR would be responsible for their longevity. Furthermore, we measured the response of RNAi-defined "Mit mutants" to additional *sdha-1*, *sdha-2* and *sdhb-1* RNAi knockdown since it has been proposed that the mechanisms underlying their longevity are not identical to those in genetic mutants (Yang & Hekimi, 2010b). Wild type animals were subjected to RNAi against genes that encode CI (*nuo-4*) and CIII (*cyc-1*) subunits, known to lead to longevity (Dillin et al., 2002; Hansen et al., 2005; Rea et al., 2007). After CI or CIII RNAi until L4, we switched the animals to RNAi against CII subunits (*sdha-1*, *sdha-2*, *sdhb-1*). The changes in life span reflected the findings in genetic Mit mutants with the strongest effect caused by *sdha-2* knockdown. These data suggest that longevity induced by either ETC subunit mutation or RNAi knockdown likely depends on SDH (*sdha-2*) activity.

Furthermore, activity of CII affects life span alterations induced via pathways

that are likely independent of mitochondria, namely IIS (*daf-2(e1370)*) and DR (*eat-2(ad1116)*). These mutants react to CII inhibition with a shortened life span, similar to wild type.

To complement our functional mutant and knockdown studies with gene expression data, we monitored transcription of both catalytic subunits, *sdha-1* and *sdha-2*, and the iron sulfur subunit *sdhb-1* during development by quantitative PCR. Most importantly, a switch from *sdha-1* to *sdha-2* occurs after L1 in wild type. *gas-1* differs from wild type by higher *sdhb-1* expression in L4 and *sdha-1* in adulthood. *isp-1;ctb-1* deviates as well by increased expression of *sdha-1* and *sdha-2* in L4. This led us to speculate that difference in expression level of these CII subunits in L4 could cause a metabolic shift, altering the life span.

Expression level changes of CII subunits prompted us to analyze NADH, SDH, and COX enzymatic activities in Mit mutants by histochemical staining as described (Paper III). SDH activity was up-regulated in all examined tissues in short-lived *gas-1* which suggests that high CII activity is required to compensate for the low electron number entering the ETC through CI, allowing development and fertility. While NADH activity remained unchanged in *isp-1;ctb-1*, SDH activity was up-regulated in the intestine and pharynx and slightly in body wall muscles. This result indeed correlates with the increased *sdha-2* expression in these animals. COX activity was significantly increased in all tissues in *gas-1* and *isp-1;ctb-1*, with the highest levels in the intestine.

UPR$^{mt}$ is proposed to be involved in regulation of mitochondrial dysfunction-induced longevity in *C. elegans* (Durieux et al., 2011). We analyzed the UPR$^{mt}$ response in Mit mutants upon CII inhibition and confirmed that UPR$^{mt}$ is up-regulated in wild type subjected to RNAi against *cyc-1, cco-1*, and *atp-5* from L4 stage onward. We furthermore tested the UPR$^{mt}$ response in genetic Mit mutants and observed that it is strongly activated from L3 stage onwards in both, short- (*mev-1*) and long-lived (*isp-1, isp-1;ctb-1, nuo-6*) mutants. We noticed that the UPR$^{mt}$ response remains active through adulthood, even though it slightly decreases with increasing age. We tested the effect of CII RNAi on the UPR$^{mt}$ response in wild type animals and found that RNAi against *sdha-1* and *sdhb-1* genes induces an UPR$^{mt}$ response specifically during larval development in wild type, while *sdha-2* deficiency did not have any effect. The up-regulation of UPR$^{mt}$ in the long-lived *isp-1;ctb-1* was not affected by RNAi treatment against various SDH subunits, even though it leads to

major decrease in lifespan. Thus, our data suggest that $UPR^{mt}$ response seems to be active in Mit mutants independently of their longevity.

We hypothesized that longevity of Mit mutants arises as a function of specific metabolic state. *clk-1(qm30)* and *isp-1(qm150)* mutants have increased pyruvate production, while the short lived mutants (*mev-1(kn1)* and *ucr.2.3(pk732)*) have increased lactate, α-ketoglutarate and dipeptide Gly-Pro (Butler et al., 2010). Therefore, we quantified changes in gene expression of the key enzymes involved in various metabolic pathways (TCA, gluconeogenesis, glyoxylate cycle, anaerobic pathways, glycolysis and lipid metabolism) by real time PCR. We observed a general increase in expression of TCA cycle enzymes in both, short-lived (*gas-1*, *mev-1*) and long-lived (*isp-1* and *isp-1;ctb-1*) mutants, as well as slight increase in glycolytic enzymes. Lipase expression was decreased, which is likely reflected by a significant increase in lipid content in all tested Mit mutants. Similarly, a decline of respiratory chain enzyme activities in heart specific TFAM knockout mice led to a global metabolic switch. Gene expression analysis in these animals showed up-regulation of glycolytic and down-regulation of fatty acid oxidation enzymes (Hansson et al., 2004).

Microarray analysis showed that long-lived genetic - *isp-1, clk-1* -, and RNAi-defined - *cyc-1* (cytochrome c subunit of CIII) - Mit mutants up-regulate the glyoxylate cycle (Cristina et al., 2009). This gave rise to the idea that the glyoxylate cycle might be a prerequisite for mitochondria-dysfunction induced longevity in the background of impaired respiration. *C. elegans* has a single, bifunctional glyoxylate cycle protein, the isocitrate lyase/malate synthase (*icl-1*), that is responsible for cleavage of isocitrate to succinate and glyoxylate (isocitrate lyase activity), and for production of malate from glyoxylate and acetyl-CoA (malate synthase activity). ETC dysfunction could impair the TCA cycle since succinate is not oxidized to fumarate. This could further lead to decreased formation of oxaloacetate, the substrate of glutamate synthesis. In order to generate glutamate, anaplerotic (refilling) pathways such as the glyoxylate cycle and fatty acid oxidation, could be activated in order to supply mitochondria with oxaloacetate and acetyl-CoA. Succinate produced by the glyoxylate pathway can then be fed back into the TCA cycle or can be transported to the cytoplasm where it would serve as substrate for gluconeogenesis. In contrast to this hypothesis, we did not observe major differences in expression of components of the glyoxylate cycle or gluconeogenesis in long-lived *isp-1* or *isp-1;ctb-1* mutants.

On the contrary, we found strong up-regulation of glyoxylate cycle enzymes but not of gluconeogenesis in the short lived *gas-1* mutant. Both, *gas-1* and *isp-1,ctb-1* mutants, had additional changes in anaerobic pathways, specifically in alcohol dehydrogenase and malic enzyme expression. In summary, these data are in agreement with previous work, showing that mutations in the ETC lead to a general response characterized by up-regulation of TCA cycle enzymes and of pathways that provide it with substrates (Falk et al., 2008).

How could the difference in longevity between *gas-1* and *isp-1;ctb-1* mutants under CII inhibition be explained? It was suggested that oxygen consumption rate might be a major determinant of hypoxic response in mammals (Chua et al., 2010). In worms, life span of long-lived Mit mutants (*clk-1* and *isp-1*) is dependent on hypoxia induced transcription factor (HIF-1) under normoxic conditions (Lee et al., 2010). Thus, the decrease in oxygen consumption could in fact mimic a hypoxic condition, further leading to HIF-1 activation. Why, however, would HIF-1 selectively affect the longevity in Mit mutants? We found that *gas-1(fc21)* mutant have decreased oxygen consumption to about 25% of wild type amounts (Paper III). Furthermore, *gas-1* has a strong increase in SDH activity, likely being a compensatory mechanism for CI dysfunction. Knockdown of CII subunits in *gas-1* mutant might decrease oxygen consumption even more, due to complete blockage of electron entry into the ETC. It has been shown that mutations in different CII subunits have opposite effects in human cell lines under normoxia. Mutations in the SDHB subunit lead to increased ROS and succinate levels, while mutations in SDHA only increase succinate level in human cell lines (Guzy et al., 2008). Similar contrasting effect upon inhibition of CII might be envisioned in *gas-1* mutant animals, too. Knockdown of SDHB could lead to an increase in ROS production in *gas-1* animals that might result in increased longevity, potentially via HIF-1 activation.

We showed that mutation in SDHC subunit (*mev-1(kn1)*) could lead to decrease in CII activity, while increasing enzymatic SDH activity (Paper III). This could lead to a high electron entry at flavin group in SDHA, but the electron removal from the flavin group might be impaired due to present mutation in SDHC subunit. Consequently, this might result in an increase in ROS production. Similarly, mutations in SDHB and SDHD might result in higher SDH activity and therefore increased ROS production. Indeed, it was observed that mutations in the SDHC and SDHB subunits increase superoxide production in *C. elegans* (Huang & Lemire,

2009; Senoo-Matsuda et al., 2001). The reason why the knockdown of SDH activity does not lead to increase longevity might be due to lack of additional ROS production because electron would not enter CII and therefore flavin group would stay oxidized. This might suggest that pseudo-hypoxic condition derived upon ETC dysfunction could result in increased longevity if at least two requirements are fulfilled, HIF-1 stabilization and certain ROS production.

Additional RNAi knockdown of CII subunits in *isp-1(qm150);ctb-1(qm189)* mutant animals significantly decreases their life span, suggesting that they strongly rely on functional CII. We found that *isp-1;ctb-1* mutant longevity is inversely affected by RNAi against *sdha-2* (CII) and *cco-1* (CIV). While *sdha-2* RNAi dramatically decreases their life span, *cco-1* RNAi increase it even more. RNAi against *sdha-2* decreased the oxygen consumption for about 10% in comparison to *isp-1;ctb-1* leaving about 40% of normal oxygen consumption measured in wild type animals. Knockdown of CIV (*cco-1*) in these mutants decreased even more oxygen consumption to about 20% of wild type amounts, thus suggesting that the longevity can be uncoupled from respiration. It has been proposed that certain level of ROS is necessary for the longevity of *isp-1;ctb-1* mutants (Yang & Hekimi, 2010a). Thus, ROS could also regulate HIF-1 stabilization in these mutants and thereby regulate their longevity. Additionally, we found that mild mitochondrial dysfunction in *isp-1;ctb-1* mutants does not lead to anaerobic reprogramming but rather to up-regulation of TCA cycle that could result in accumulation of succinate. It was shown that also succinate can mediate HIF-1 stabilization. Excess succinate can leak to the cytoplasm and directly inhibit prolyl-hydroxylases (PHDs). Consequently, this would lead to HIF-1 stabilization and up-regulation of stress responses in the cell (Pollard et al., 2003). Lastly, the question what determines the longevity of *isp-1;ctb-1* mutants remains open, but we know now that CII activity is of vital significance for their longevity.

# 4. Concluding Remarks

Our work had two tightly linked objectives:

1) Development of methods for analysis of mitochondrial DNA level, dynamics, morphology and electron transport chain enzyme activities on tissue level in *C. elegans* (Paper I and III)

2) To further elucidate the role of mitochondria during development and ageing of *C. elegans* (Paper II and IV).

We have developed a number of new strategies that we believe, enrich the toolbox of techniques to study the mitochondrial replication, transcription, mitochondrial morphology and metabolic changes on a single-tissue level. We were able to measure mtDNA copy number in single animals and use this to record mtDNA replication throughout *C. elegans* development. Our results indicate that mtDNA replication starts at L3 larval stage and that it mirrors germline proliferation. This is in agreement with the finding in mice that mtDNA replication is paused during pre-implantation period of an embryo, indicating that *C. elegans* is indeed a well-suited model to characterize mechanisms of mtDNA inheritance and maintenance in both postmitotic and proliferative tissues. In addition, we optimized a method for *in vivo* imaging of the dynamic mitochondrial network by mechanical immobilization of the animals, circumventing any potential side effect of routinely used drugs. This functional *in vivo* examination was complemented by a palette of *in situ* studies in natively frozen worm sections that are advantageous over whole-mount preparations for histochemistry. They simultaneously retain morphology, enzymatic activity, lipids carbohydrates, and antigenicity. Thus, they allow for quantification of various indicator reactions *in situ* on tissue level. We could now measure significant metabolic changes and record significant differences between tissues types, similarly to mammalian systems.

The most exciting finding in POLG deficient worms was the ability of homozygous *polg-1* mutants animals to have a normal embryonic and larval development despite severe mtDNA depletion, contrasting other organisms where POLG deficiency leads either to embryonic (mice) or larval (*Drosophila*) arrest. We propose that this occurs due to high maternal mtDNA inheritance that ensures normal development. POLG deficiency further leads to severe mtDNA depletion and morphological defects in the hermaphrodite gonads, the main site of mtDNA

replication in worms, while it only modestly affects somatic tissues. In the late adulthood, we detected slight changes in somatic tissues of *polg-1* deficient animals, a likely increase in mitochondrial fusion, presumable as a compensatory mechanism activated upon mitochondrial dysfunction. mDNA copy number is an essential an limiting factor in worm development, requiring a minimum amount of mtDNA to be supplied in the zygote embryonic development. Last, we demonstrated that the mtDNA copy number in worms shows great plasticity as a response to the different environmental stimuli.

We confirmed that longevity of Mit mutants is severely affected by additional inhibition of the ETC, indicating the importance of intact ETC function for their long life. We concluded that SDH activity is one of the prerequisites for life span prolongation of the Mit mutants. CII divergently regulates the longevity of short-lived *gas-1* and long-lived *isp-1;ctb-1* mutants. Moreover, SDH inhibition influences the longevity of *isp-1;ctb-1* mutants, even if occurs only during adulthood. While it had been hypothesized that FR activity might be the major regulator of longevity in Mit mutants, our study clearly shows that this is not the case. On the contrary, we found that FR activity is necessary for the life span of wild type animals, while it only has a minor effect on the life span of the long-lived *isp-1;ctb-1* mutant. Our data further indicates that longevity in Mit mutants does not entirely depend on respiration. We confirmed, in agreement with the previous studies, that Mit mutants are characterized by a specific metabolic profile, general up-regulation of the TCA cycle, glycolysis and anaerobic pathways. However, we showed that long-lived genetic Mit mutants do not have up-regulation of glyoxylate cycle as previously thought, while short-lived genetic Mit mutants do.

Recently, understanding of pathways underlying mitochondrial dysfunction induced longevity in *C. elegans* has emerged. Still, many questions remain to be answered, especially if and how these longevity pathways are linked. Nevertheless, characterization of *C. elegans* ageing and clearer understanding of basic principles behind may at some point lead to healthier life in humans.

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